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THE BRITISH JOURNAL OF
EXPERIMENTAL PATHOLOGY

VOL. XXIV AUGUST, 1943 NO. 4

THE RELATION BETWEEN AGE, STRUCTURE, AND AGENT CONTENT OF ROUS No. 1 SARCOMAS.

J. G. CARR.

From the Institute of Animal Genetics, Edinburgh 9.

Received for publication May 22, 1943.

It is well known that extracts, or other preparations of cell-free agent from the Rous No. 1 sarcoma, as well as other filterable fowl tumours, vary very much in the amount of active agent contained in them, even when the same technique is scrupulously followed in each experiment (Rous and Murphy, 1914 ; Gye and Andrewes, 1926 ; Baker and McIntosh, 1927 ; Cramer and Foulds, 1930 ; Gye and Purdy, 1930, 1931 ; Doerr, Bleyer and Schmidt, 1932 ; Amies and Carr, 1939 ; Carr, 1942). Not only may the agent be totally lacking, but the extract may even have an actively inimical action upon added agent—the "inhibitor action" (Gye and Purdy, 1931 ; Sittenfield, Johnson and Jobling, 1931 ; Murphy and Sturm, 1932). As the causes of these variations have not been understood, their occurrence has seemed capricious in nature. Thus Cramer and Foulds (1930) remark, "It is impossible to predict whether a particular Rous sarcoma No. 1 will yield a completely inactive filtrate."

In connection with studies upon the cause of the resistance of a line of fowls to the development of Rous No. 1 tumours, it became necessary to undertake a study of these phenomena. It was found that not only could this prediction be made, but that the activity of any agent preparation could be foretold with reasonable accuracy in most experiments.

Determinations of the amount of agent per gramme of tumour were available for 61 tumours, together with records of size, rate of growth and structure. The hosts were all Brown Leghorns of the Institute flock, aged about 6–9 weeks at the time of inoculation of the tumour material. Most of them were males, and the majority had been injected with cell-free material. Otherwise they had little in common. They were from various inbred lines and bore single or multiple tumours, due either to injection of plain tumour extract, or mixtures of agent with antiserum, inhibitor, or other materials. The activity was variously determined upon extract, or material recovered after various methods of processing. The time of extraction varied from 1 hour to over 30 hours. In all experiments hydrocyanic acid was present as an inhibitor of oxidation. Agent content was determined by injecting suitable decimal dilutions of the final product into two or three susceptible chicks. In spite of the increased chances of altered activity thus allowed by modifications of technique, the activity showed a

rather consistent variation with the age of the tumour alone. As shown in the figure, all tumours processed up to the 13th day after inoculation of the host contained 10^6 or more agent particles per gramme of tumour. Between the 14th and 22nd days the activity was often less than this, but never below 10^5. Later than this, the activity was less than this in about half the material taken. No active agent was found after the 40th day, while extracts of all tumours taken before this time contained some agent. Considering the heterogeneous nature of the material, and the crude approximation to the agent content obtained by titrating decimal dilutions, the scattering of the points is not very extreme, and it is seen that a fairly reliable indication of the extracted agent can be obtained from a consideration of the age of the tumour.

On the other hand, the structure of the tumour, which has often been considered to give an indication of the amount of agent obtainable in extracts (Gye and Andrewes, 1926 ; Gye and Purdy, 1930, 1931), was, in this series, found to be of very slight value.

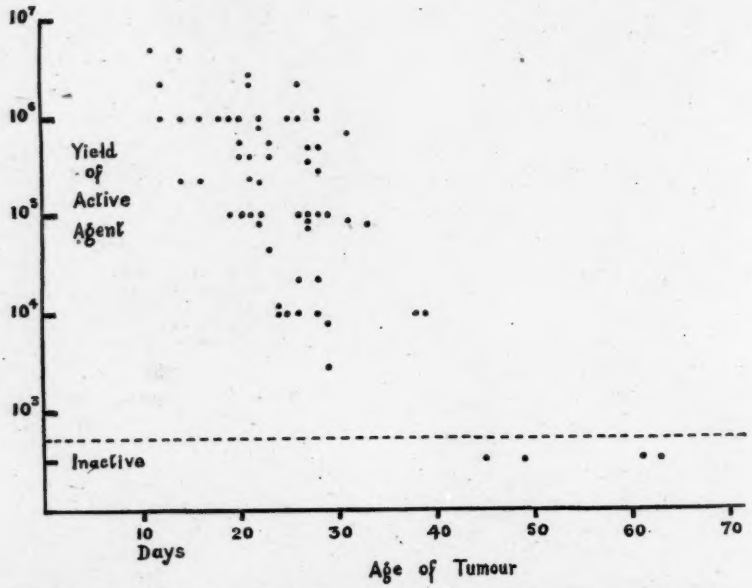

FIG. 1.—The amount of agent extracted per gramme of tumour from tumours of varying ages.

It was noted, for example, that three of the tumours of 16–22 days' growth were slowly growing, and white or yellow and rather hard when taken, yet their agent content was as high as that of rapidly growing tumours of the same age. This failure of structure and appearance to give any reliable clue to the content of agent extractable from the tumour was noted throughout the course of this work. As an illustration of the type of finding in the case of tumours of recent growth, the following experiments are given in detail :

Experiment 1.—Fowls 652 and 653 were inoculated in both breasts and both legs with a Rous extract.

Fowl 652 proved extremely susceptible to the Rous tumour agent, and was bearing large tumours when killed on the 16th day after inoculation. These were soft, pink, fungating, very malignant growths, in which a good deal of haemorrhage had occurred

—in fact, they were typical examples of this tumour in its most virulent form. The tumour in the right breast was processed by the method of Amies and Carr (1939), and yielded 10^5 agent particles per gramme of tissue.

Fowl 653 was much more resistent to tumour growth. At the end of 19 days after inoculation the tumours were only about one-third of the size of those of 652 at death. They were found to be compact, very yellow, quite firm, and were not invading the surrounding muscles in the marked fashion noted in Fowl 652. Only one tumour showed any signs of haemorrhage, and this was in a tumour in the leg, and was believed to be due to rupture of a small blood vessel caused by muscular action immediately after death. The tumour corresponding to that used in Fowl 652 was processed by the same method, and the same yield of tumour agent obtained.

Experiment 2.—One of the animals used to titrate the agent of Fowl 652 grew rather small and slow tumours. The bird was killed on the 14th day after inoculation, and the largest tumour taken. It was pale yellow, firm, and with little invasive tendency. The centre was more yellow and was discarded as being probably necrotic. The remainder of the tumour was processed by the same method as used in the previous experiment and yielded 5×10^6 agent particles per gramme of tissue.

Experiment 3.—Fowl 530 was inoculated with varying dilutions of a routine agent preparation. Tumours appeared in all sites, and grew quite rapidly. The largest was noted to be unusually soft, and when the bird was killed on the 16th day after inoculation it was found to consist of soft fragments of tumour tissue growing from the edge of the tumour or lying loose in a semi-fluid matrix. Portions of the tumour tissue were removed and processed by the same method as before, and the product yielded 5×10^6 agent particles per gramme of tissue.

Similarly, it was found that tumours which grew rapidly, and had the characteristic appearance of such tumours, did not necessarily contain a large amount of extractable agent. One such case will be described in detail:

Experiment 4.—The agent-induced tumours of Fowl 640 grew very slowly at first (a companion bird inoculated with the same material was killed with very large tumours after 22 days). About 40 days after inoculation the tumours began to increase in size very rapidly, and the animal was killed on the 49th day bearing very large tumours, all soft, fungating, haemorrhagic, very malignant, and typical of the rapidly-growing type of Rous No. 1 tumours. A plain 10 per cent. cell-free extract of this material was found to be devoid of any agent activity.

Two of the other tumours of more than 40 days' growth were also the result of a similar spurt of growth, and had a similar structure, yet no active agent could be extracted from them. Appearance seems to be of value only in indicating that a large tumour of the slowly-growing type must be an old one, and hence only a small yield of extractable agent is to be anticipated.

It was also noted that the results gave no support to the claim (Cramer and Foulds, 1930) that slowly-growing tumours result from "attenuated agent" obtained from tumours of slow growth and low agent content. The rate of tumour growth appeared to be determined by the susceptibility of the host (as seen, for example, in Experiments 1 and 4), and neither the source nor the amount of agent seemed to have any influence. It has been shown that this varied susceptibility of the Institute flock is often due in part to inherited factors (Greenwood, 1940; Carr, 1943; and unpublished findings). Many other experiments showing that the rate of growth of the tumour is dependent upon the susceptibility of the host and not on the source of the tumour agent are to be found in these papers.

This concept of variation with the age of the tumour will also account for the non-filterability of the recurring tumours previously described (Carr, 1942). Although

several of these were rapidly-growing sarcomas, they developed several months after the initial stimulus of tumour material was first experienced by the fowl, and some tumour material must have been present, though latent, until the recurrence of the tumours. These tumours should therefore be considered as equivalent to tumours of several months' duration, and the absence of any agent in the extracts is in agreement with the suggestion that activity and age are inversely related.

So far only animals inoculated at the age of 6–9 weeks have been considered. It requires only a slight extension of the concept described above to deal with the case of animals inoculated at a more advanced age. While the serum of young chicks has no anti-Rous activity (Amies, 1937), older birds often contain " naturally-occurring " antibodies to the Rous No. 1 agent. Whether this is due to agent remaining in the bird without causing tumours, as occurred in the experiments of Rous, Murphy, and Tytler (1912), Pentimalli (1924), and in the case of the recurring tumours just mentioned, or to a related virus with a common antigen, or to a heterologous antigen, is a matter for further investigation. But it is reasonable to ante-date the " tumour stimulation " in these cases to a period before the inoculation of the experimental material, and thus to expect that such fowls would be especially prone to bear tumours yielding inactive extracts. This is well known to be the case. Furthermore, as commercial hatching is usually carried out almost exclusively in the spring, it might be expected that workers who use animals from such sources would find that the activity of tumour extracts would fall progressively throughout the year, but increase again in spring. The variations reported by Gye and Andrewes (1926) and by Fränkel (1930) are significant in this connection.

DISCUSSION.

In this work only the Rous No. 1 sarcoma has been considered. It is seen that the age of the tumour is the most important factor in determining the activity of the extract prepared from it. Many other factors will, of course, modify this relationship. In particular, very young chicks seem to be abnormally responsive to Rous agent (" haemorrhagic disease " of Duran-Reynals, 1940), and the effect of age may be less in such animals. A limited number of experiments on such animals have suggested that this is so, but the data are insufficient for a definite conclusion to be drawn.

The decrease in activity is probably due to an inhibitory substance elaborated by the fowl or the tumour, as in certain types of experiment the agent can be separated from such a material, e.g. by high-speed centrifugation or absorption (Sittenfield, Johnson and Jobling, 1931 ; Murphy and Sturm, 1932 ; Claude, 1939). Use of such techniques should confuse the relationship demonstrated above. Though in this work no fowls of the N-S strain bred for decreased response to Rous agent are included, it was found that data from them fitted into the scheme quite readily, and that activities were easily predicted by considering the age of the tumour alone.

It is the experience of workers using other filterable fowl tumours that filterability varies in a fashion similar to that found for the Rous No. 1 tumour, with some modifications due to the special characteristics of the tumour strain employed. A complete discussion of this, and of the mechanisms involved, is deferred until the experiments on the " inhibitor " are described. But it seems desirable to point out that this variation with age due to an inhibitor seems to be common to all tumours induced by a virus-like intracellular agent. Therefore experiments designed to prove the existence of such agents are more likely to succeed if very young grafts are used. The increase in tumour material obtained with older grafts may not compensate for the reduction

in the amount of tumour agent which may result. It also follows that a slowly-growing tumour, though bearing a filterable agent, will be non-filterable for the greater part of its life in any host.

SUMMARY.

The amount of active agent extracted from Rous No. 1 tumours was inversely proportional to the duration of growth in the host. After 40 days all tumours were non-filterable ; before then all contained some agent. The appearance of the tumour bore no relation to the amount of agent obtained in extracts.

All expenses in connection with this work were borne by the British Empire Cancer Campaign.

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PROLONGED ANTIBODY PRODUCTION FOLLOWING RECOVERY OF FOWLS FROM ROUS No. 1 SARCOMA.

J. G. CARR.

From the Institute of Animal Genetics, Edinburgh 9.

Received for publication July 10, 1943.

As is well known, there are two types of immunity following virus infection ; after certain diseases there is a transient period of immunity, while others confer a very prolonged, sometimes life-long immunity. It is usually considered that the latter type of immunity results from the virus remaining latent in the host, corresponding somewhat to the " carrier " stage of bacterial infections, though rigorous proof of the presence of the virus is not always available. It has been recognized from the beginning of work on the filterable fowl tumours that certain fowls may be resistant to the inoculated agent, and that if they recover from the infection they are immune to further inoculation (Rous and Murphy, 1914). The exact duration of this immunity has not been studied, and it remains a matter of controversy how far this immunity is due to humoral antibody, and how far it is due to other types of resistance. Though such fowls have often been used for immunological studies on the fowl tumour viruses, workers seem to have had little faith in the ability of the fowl to maintain a high antibody titre, and have usually preceded their investigations by a series of immunizing injections of tumour material.

In the course of work which involved keeping fowls for a considerable length of time after they had recovered from a single injection of Rous No. 1 virus, it was found that tumours would sometimes recur in these birds many months after the first induced tumours (if any) had vanished, and Rous No. 1 virus was recovered from grafts of these tumours (Carr, 1942). It thus became necessary to postulate the existence of the Rous virus in a latent form in these birds, and consequently it might be anticipated that such birds might have constant high antiviral antibody titre in their serum. Investigations were therefore carried out to see if this was the case, and to obtain information as to how often this would be found, thus obtaining information as to what proportion of birds have the virus remaining latent in the tissues after recovery from the tumours.

It was found without exception that sera obtained from birds which had recovered from induced Rous No. 1 tumours some time previously possessed strong virus-neutralizing activity when tested *in vitro*, irrespective of the time which had elapsed between taking the blood sample and the inoculation with tumour material. Of many tests performed, the following four are selected as examples because they belonged to a small group of fowls kept in individual cages and under constant observation. All were females which had been tested for susceptibility to Rous agent at the age of six weeks by the method previously described (Carr, 1942).

Experiment 1.—Fowl 0.135. For previous history, which included a temporary recurrence of the Rous tumour, see Carr (1942).

Fowl 0.1262. Tested 13.viii.41, the largest dose of agent containing over 1000

minimal infective doses (M.I.Ds.). Small tumours appeared in the breasts and soon regressed.

Blood was withdrawn from these two fowls and from a young normal chick on 6.viii.42, and the sera removed from the clot next day. The serum, or a 1/10 dilution of it, was mixed with an equal volume of a cell-free Rous agent suspension diluted to such an extent that 0·5 ml. of the mixture contained about 50 M.I.Ds. of agent. The mixture was incubated for two hours at 37° and then left in the cold room overnight. Next day the activity of agent in the various mixtures was determined by inoculating 0·5 ml. into the breast or leg muscles of susceptible chicks as shown in the table.

Table Showing Neutralizing Action of the Sera taken from Birds which had Recovered from Rous Tumours.

Inoculum.						Fowl 525.	Fowl 526.
Rous agent	+ normal	fowl	serum	.	.	+++	+++
,, ,,	+ 0.135	,,	,,	.	.	−	−
,, ,,	+ normal	,,	,,	× 1/10	.	+++	+++
,, ,,	+ 0.135	,,	,,	× 1/10	.	+	++
						Fowl 527.	Fowl 528.
,, ,,	+ normal	,,	,,	.	.	+++	++
,, ,,	+ 0.1262	,,	,,	.	.	−	−
,, ,,	+ normal	,,	,,	× 1/10	.	+++	++
,, ,,	+ 0.1262	,,	,,	× 1/10	.	−	+

The size of the tumours when the birds were killed is indicated by the number of + signs.

Experiment 2.—Fowl 0.625. Tested 30.v.41, the largest dose containing about 1000 M.I.Ds. A small tumour appeared at the site of the largest dose, but soon regressed. The subsequent history of the bird was in no way remarkable. No recurrence of the tumours was noted. A blood sample was withdrawn 27.x.42, and tested by the same technique as in Experiment 1. Serum diluted to 1/4 and 1/40 completely inactivated 100 M.I.Ds. of agent contained in an equal volume of suspension.

Experiment 3.—Fowl 0.619. Tested at the same time, and in the same way, as 0.625. Tumours appeared in both breasts, and lasted rather longer than in 0.625 before regressing. A blood sample was withdrawn 13.iv.43, and 3 ml. of the serum obtained completely neutralized the activity of 30 ml. of agent suspension containing over 10,000 M.I.Ds. As confirmatory evidence that this inactivation was due to neutralization by antibody and not to another type of virus destruction, the mixture of virus and serum was centrifuged and active virus recovered from the deposit. That this separation of agent and antibody is also found with serum obtained from immunized fowls was previously shown by Amies and Carr, 1939.

DISCUSSION.

The neutralizing power of these sera was quite as strong as that of sera taken from birds which had been submitted to a preliminary course of immunization. Exactly how the virus, presumably having an intracellular existence, can induce the formation of antibodies in serum which does not penetrate the cell-wall is not a problem peculiar to the virus of the Rous No. 1 sarcoma. There is no evidence that a diffusible specific soluble substance is produced as is the case with vaccinia ; but a similar problem also

exists in the case of many other virus infections, and is more likely to be solved by workers who use material more easily handled than are the fowl sarcoma agents.

The demonstration of active antibody in these fowls can, by analogy with other virus diseases, be regarded as proof of the presence of tumour virus, though tumours are not produced. As virus has been recovered from birds over a year after inoculation (Carr, 1942), it was known that it could remain latent in some birds. In the present investigation high antibody titres were found in all tested birds, so it would appear that this idea of latent virus must be extended to all birds recovering from Rous No. 1 tumours. The manner in which the virus can exist in the bird without producing tumours is unknown; but it is known to remain in the tissues in a similar inactive way after intravenous inoculation (Rous, Murphy and Tytler, 1912; Pentimalli, 1924) and when disseminated from growing tumours (Mellanby, 1938). The analogy of this condition to the "milk factor" of mouse mammary tumours is apparent.

The importance of this latent fowl tumour virus cannot yet be evaluated. Though birds infected with virus-induced tumours will not produce immediate epizootic outbreaks of neoplasms in a flock (Rous, Murphy and Tytler, 1912), there is no evidence that the virus is not transmitted in this latent form, either between fowls or *via* the egg. Should this occur, instead of an immediate outbreak of tumours, it would seem more reasonable to expect the disease to appear in a similar fashion to the manner in which it appeared in the case of the recurring tumours previously described, *i.e.* isolated occurrences of the tumour at irregular intervals in a proportion of the birds. The high incidence of neoplastic diseases in most flocks suggests that such an aetiology is not altogether improbable, and it is very unfortunate that there is no reliable evidence as to the identity or otherwise of viruses responsible for neoplasms in isolated poultry flocks.

SUMMARY.

Fowls tested one to two years after recovery from Rous No. 1 tumours all possessed a high content of neutralizing antibodies to the Rous No. 1 agent in their serum. This is regarded as evidence that the virus has remained in a latent form in the tissues of these birds.

All expenses in connection with this work were borne by the British Empire Cancer Campaign.

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INVESTIGATION INTO THE PRODUCTION OF BACTERIOSTATIC SUBSTANCES BY FUNGI. PRELIMINARY EXAMINATION OF A SECOND 100 FUNGAL SPECIES.

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From the Mycology Laboratory, University Department of Botany, Oxford.

Received for publication May 31, 1943.

THE following results are based on work which directly continues in scope, objective and method that already published (Wilkins and Harris, 1942). The examination of a further 100 fungal species has been carried out as before, though increased experience of the influence of medium composition, etc., has perhaps led to a more decisive opinion as to which are positive and which negative. The fungi were grown in various media and in each case the culture liquid was tested for its bacteriostatic properties, by the method previously described (Wilkins and Harris, 1943). In the following list a positive sign indicates that inhibition has been effected by at least one of the culture liquids, though the degree of the inhibition is not stated. A negative sign implies that no inhibition has been obtained at any time, or that the degree of inhibition is so slight as to be relatively insignificant. Each liquid was tested against the three organisms—*Bact. coli*, *Staph. aureus* and *Pseud. pyocyanea*. In the case of those fungi which possess + and − strains, these strains were grown separately and also together, but with no difference in the result. Many of the cultures were obtained from the Lister Institute and, in these cases, the " Lister Number " is given. This number is important in distinguishing between the different strains of the same species.

LIST OF FUNGI EXAMINED.

PHYCOMYCETES.

Positive.

	Bact. coli.	*Staph. aureus.*	*Pseud. pyocyanea.*
Phytophthora erythroseptica Pethybridge . . .	+	+	−

Negative.

Absidia glauca Hagem. + and −; *Basidiobolus ranarum* Eidam; *Chaetocladium Brefeldii* v. Tieghem & le Monnier + and −; *Cunninghamella echinulata* (Thaxter) Sacc. + and −; *C. elegans* Lendner + and −; *Mortierella reticulata* v. Tieghem & le Monnier; *Mucor hiemalis* Wehm. + and −; *M. mucedo* Linn. + and −; *M. sexualis* G. Smith; *Phycomyces nitens* (Kunze) v. Tieghem & le Monnier + and −; *Phytophthora cactorum* (Leb. & Cohn.) Schroet; *Rhizopus nigricans* Ehrenberg + and −; *R. sp.* (Homothallic); *Sporodinia grandis* Link; *Syncephalastrum racemosum* (Cohn.) Schroet; *Thamnidium chaetocladioides* Bref.; *T. elegans* Link; *Zygorhynchus Moelleri* Vuill.

Ascomycetes.

Positive.

	Bact. coli.	*Staph. aureus.*	*Pseud. pyocyanea.*
Chaetomium convolutum Chivers	—	+	—
Neocosmospora vasinfecta E. Smith	—	+	—
Sclerotinia minor Jagger	+	+	—

Negative.

Chaetomium elatum Kunze & Schmidt; *Hansenula anomala* (Hansen) Syd.; *Lachnea cretea* (Cke.) Phill.; *Melanospora parasitica* Tul.; *Neurospora sitophila* Shear & B. O. Dodge + and —; *N. tetrasperma* Shear & B. O. Dodge + and —; *Saccharomyces cerevisiae* Hansen; *S. ellipsoideus* Hansen; *S. Marxianus* Hansen; *S. Pastorianus* Hansen; *Ustulina vulgaris* Tul.; *Xylaria longipes* Nitschke; *X. mali* Fromme; *X. polymorpha* (Pers.) Grev.

Penicillia.

Positive.

	Bact. coli.	*Staph. aureus.*	*Pseud. pyocyanea.*
P. aurantio-violaceum Biourge 3943	—	+	—
P. baculatum Westl. 3956	—	+	—
P. brevi-compactum Dierckx. 3568	—	+	—
P. caseicolum Bain. 1717	—	+	—
P. Charlesii G. Smith 4154	—	+	—
P. chrysogenum Thom. 3882	+	+	+
P. chrysogenum Thom. 3953	+	+	—
P. crustosum Thom. 4002	+	+	—
P. Daleae Zal. 3800	+	+	—
P. digitatum Sacc. 3498	+	+	—
P. digitatum Sacc. 3949	+	—	—
P. Duclauxii Delacr. 587	—	+	—
P. expansum (Link.) Thom. 5612	+	+	+
P. frequentans Westl. 5613	—	+	—
P. glabrum Wehm. 3944	+	+	—
P. glabrum series Thaysen 606	+	+	+
P. gladioli Machacek 5614	+	+	+
P. Hagemi Zal. 3565	+	+	—
P. Herquei Bain. & Sart. 1721	+	+	—
P. hirsutum Dierckx. 3566	—	+	—
P. janthinellum Biourge 5616	+	—	—
P. lanoso-coeruleum Thom. 3973	+	+	—
P. lanosum Westl. 3799	+	+	—
P. meleagrinum Biourge 3958	+	+	—
P. ochraceum (Bain.) Thom. 3971	—	+	—
P. olsoni Bain. & Sart. 5620	—	+	—
P. pfefferianum Wehm. 3936	+	+	—
P. Raistrickii G. Smith 4153	+	+	+
P. spinulosum Thom. 5625	+	+	+
P. tardum Thom. 1719	—	+	—
P. terrestre Jensen 3981	—	+	—
P. thomii Maire 5626	+	+	+
P. viridicatum Westl. 3998	+	+	—

Negative.

P. camemberti Thom. 582; *P. chrysogenum* Thom. 3357; *P. commune* Thom. 3974 and 5610; *P. corymbiferum* Westl. 4039; *P.* (*Scop.*) *danica* v. Beyma. 5811; *P. digitatum* Sacc.

3947 and 3948 ; *P. Herquei* Bain. & Sart. 4044 ; *P. italicum* Wehm. 3995 and 3996 ; *P. kiliense* Weidermann 4041 ; *P. lanosum* Westl. 5617 ; *P. Melinii* Thom. 5619 ; *P. pallidum* G. Smith 4151 ; *P. patulum* Bain. 1722 ; *P. pfefferianum-spinulosum* series 3940 ; *P. terrestre* Jensen 3978, 3979 and 6315 ; *P. viridicatum* Westl. 4000.

From the results of both this and the previous paper is appears that the Aspergilli and the Penicillia are the most promising group of fungi yet tested. Allowing for the limited numbers examined it seems that the Phycomycetes, Ascomycetes and Fungi Imperfecti (other than Aspergilli and Penicillia) are, under present conditions of experimentation, less likely to produce bacteriostatic substances of any significance. It may be mentioned that some 800 Basidiomycetes have been tested but, until the investigation is further advanced, no statement on the results can be made.

The tests also indicate that the results from any given species are not conclusive as different strains of a species may react very differently, e.g. of the four strains of *P. chrysogenum* already tested, three were positive in widely differing degrees, while one was negative. This fact will tend to account for the variation in results from different investigators who give the specific name only; and to explain the importance which we attach to the identification of the actual strain used.

Acknowledgments as in the previous paper.

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MULTIPLE MESENTERIC SARCOMATA IN RATS FOLLOWING INGESTION OF HEATED LARD.

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ROFFO (1938, 1939) has described a variety of tumours of the alimentary canal and its adnexa in rats fed on a diet of bread and milk, to which various fats heated to 350° C. were added. Among 200 rats so fed, 5 developed intraperitoneal and mesenteric tumours, associated with sarcoma of the liver ; in over 1000 control animals kept during the same period no such lesions were observed.

The present report deals with mesenteric tumours which occurred in the course of experiments designed to test Roffo's views on the carcinogenicity of heated fats. Altogether, among 71 of our rats which survived more than 300 days, 4 died with multiple mesenteric tumours. The gastric lesions which occurred in our earlier experiments have aleady been described (Beck and Peacock, 1941).

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EXPERIMENTAL RESULTS.

Groups 1–4 consisted of male Norwegian hooded rats, which were fed on an adequate diet of rat-cake (Beck and Peacock, 1941), plus greens once a week, and 20 c.c. milk and 0·5 c.c. cod liver oil per rat per week.

Group 1.—Lard heated at 350° C. for one hour was added to the diet of 50 rats; the average weekly consumption was about 5–7 g. per rat. Among 40 rats surviving 372 days or more, 2 died with multiple mesenteric sarcomata, 1 on the 372nd and the other on the 426th day. In the first case there was no obvious primary tumour. Multiple tumours infiltrated the intestine and spleen. Histologically the tumours were reticulo-endotheliomata. The second rat had a tumour in the liver as well as multiple mesenteric nodules, and deposits in the thymus and in the connective-tissue capsule of the left suprarenal gland. Histologically, the tumours were spindle-cell sarcomata. Neither of these rats was infested with *Cysticercus fasciolaris*, but three other rats in this group were so infested, but had no associated tumour.

Group 2.—Lard heated at 220° C. for 12 hours was added to the diet of 36 rats. The average consumption was about the same as in Group 1. Of 18 rats surviving 367 days, one died on this day with multiple mesenteric sarcomata. There was no obvious primary tumour in this case; the histology was that of an anaplastic spindle-cell sarcoma.

Group 3.—Lard heated at 220° C. for four hours was added to the diet of 50 rats. Two rats in this group were infested with cysticerci; one of these had a tape-worm in the jejunum and a single mesenteric tumour about 1 cm. in diameter in the corresponding segment of mesentery. This rat died 537 days after the start of the experiment. The tumour was attributed to the presence of the tape-worm.

Group 4.—For control purposes unheated lard was added to the diet of 16 rats. They were only allowed to consume about the same amount of fat as those in the experimental groups, but would have taken more. One rat in this group was infested with *Cysticercus fasciolaris*. No tumours occurred in this group.

Groups 5–7 consisted of male Wistar rats which were fed on a restricted diet of white bread (*ad lib.*) plus 30 c.c. milk per rat per week. (After 344 days peeled boiled potatoes were substituted for white bread, which was no longer obtainable.)

Group 5.—Lard heated at 350° C. for 1 hour was added to the diet of 20 rats. Of 13 rats surviving 301 days, one died on that day with multiple mesenteric sarcomata not associated with cysticercus infestation. No obvious primary tumour was found; death was due to peritonitis attributable to a perforation of the stomach by sarcomatous invasion. The nodules showed the histology of spindle-cell sarcoma. Two rats in this group were infested with *Cysticercus fasciolaris*; one of them died on the 365th day with a liver sarcoma associated with a cysticercus; there were no metastases. Four other rats developed multiple ulcerated papillomata in the fore-stomach, discovered *post-mortem* between the 276th and 432nd days of the experiment.

Group 6.—Lard heated at 220° C. for 12 hours was added to the diet of 20 rats. Two rats in this group were infested with cysticerci, and in one of them, which died on the 434th day, a sarcoma in the liver enclosed the cysticercus. This tumour was a spindle-cell sarcoma. Secondary deposits were found in the diaphragm, omentum, and mesentery. The same rat, and also another free from infestation, which died on the 268th day, had ulcerated papillomata of the fore-stomach.

Group 7.—Unheated lard was added to the diet of 8 rats, as controls. One of these rats was infested with cysticercus, but showed no tumour.

Thus, altogether three cases of multiple mesenteric sarcomata occurred in the 53 uninfested rats fed with lard heated at 350° C., and one in 18 surviving rats fed with lard

heated at 220° C. No such tumours occurred in the controls. Only one case of multiple mesenteric sarcomata of apparently spontaneous origin was observed by us in a series of 300 *post-mortem* examinations of rats, not infested with tape worms, in other experiments not connected with heated fats.

DISCUSSION.

In discussing multiple intraperitoneal tumours in rats, it is necessary to exclude infestation with *Cysticercus fasciolaris* before attributing such tumours to any other factor. The association between this parasite and the induction of tumours in rats was first reported by Borrel (1906), and was later confirmed experimentally by Bullock and Curtis (1924, 1926). Cysticerci in the liver frequently give rise to spindle-cell sarcomata in rats but not in mice, though the parasite is the same in both species. This curious fact is the subject of further experimental study. Such tumours have occurred sporadically in our experiments. They may give rise to widespread intraperitoneal metastases, in which, of course, the association with the parasite is not obvious. Indeed, it is necessary to incise all liver tumours in rats before *Cysticercus fasciolaris* infestation can be excluded. Roffo (1938, 1939) has described multiple intraperitoneal tumours, apparently secondary to large liver tumours, in rats fed with irradiated cholesterol or with heated fat, the effect of which he attributes to an oxidation product of cholesterol, but in over 1000 control rats up to 24 months of age kept in the same animal house he failed to observe any such tumours. Although he does not discuss *Cysticercus fasciolaris* as a possible etiological factor in his experiments, it would seem to be excluded by the absence of tumours in so large a number of controls. In three of our four cases of multiple mesenteric tumours in uninfested rats no liver tumour was present ; in the fourth a small liver tumour was present, but it was not possible to determine which of the tumours was primary. Tumours associated with cysticerci occurred in three out of eleven infested rats in this experiment. As only one case of multiple mesenteric sarcomata of spontaneous origin was observed by us in a series of 300 *post-mortem* examinations of uninfested rats in other experiments, the occurrence of these multiple tumours in animals fed with heated fats seems to be significant, despite the relatively small numbers of animals so far employed. The possibility that the multiple mesenteric tumours in Roffo's experiments and in ours were due to a chemical carcinogen therefore merits further consideration. Spindle-cell tumours have been induced in rats, but not in mice, by subcutaneous injection of lard, heated to 350° C., or unheated (Burrows, Hieger and Kennaway, 1936) ; spindle-cell sarcomata were induced in mice by subcutaneous injections of cotton-seed oil heated to 350° C. (Beck, 1941), and of sesame oil heated to the same temperature (Steiner, Steele and Koch, 1943). As most of the known chemical carcinogens are fat-soluble, it is to be supposed that they would follow the usual route of absorption for fat-soluble substances. In order to study the behaviour of a known carcinogen added to the diet, we fed rats with heated fat containing 2 per cent. 3 : 4-benzpyrene, and observed their organs at various times thereafter by direct ultra-violet fluoroscopy. Animals killed from 1–4 hours after the ingestion of benzpyrene in this way showed fluorescence of the oesophagus, stomach and variable lengths of small intestine, and of the lacteals and cysterna chyli. It was possible to aspirate the fluorescent chyle, and to demonstrate in it spectographically the presence of unchanged benzypyrene. During the same period the common bile-duct showed a paler blue fluorescence, proving that the mechanism of excretion originally described after intravenous injection of benzpyrene (Peacock, 1936) was taking place. The submucous connective tissue and the lacteal and vascular capillaries appear to be the first mesoblastic tissue to be exposed to the

action of a fat-soluble carcinogen administered in the diet. It is possible that the prolonged exposure of these mesoblastic tissues to a carcinogen may induce multiple primary tumours in somewhat the same way that multiple papillomata of the skin can be induced by repeatedly painting it with a carcinogen. The fact that three of our four tumours were spindle-cell sarcomata suggests that connective tissue of fibroblastic origin may be more susceptible to the action of a potential carcinogen in heated fat than is the endothelium. Carcinogenic action at a small distance from the point of maximum concentration of an injected carcinogen has frequently been observed by us (Peacock, 1933, and Peacock and Beck, 1938), and it seems possible that a fat-soluble carcinogen could permeate the thin walls of the lacteals and so come in contact with surrounding areolar tissue in the mesentery. It should be noted, however, that in direct fluoroscopic observation of animals fed with benzpyrene the most striking fluorescence was seen in the intra-abdominal adipose tissue, corresponding with a vascular distribution, and that direct leakage from the lacteals was not observed. The primary site of induction of spindle-cell sarcomata may therefore be in the submucous connective tissue of the small intestine. Some examples of perforation of the intestine by invasion with spindle-cell sarcoma might be taken to support this view. Further work will be necessary before the carcinogenicity of fats heated to about 350° C. can be definitely assessed, but the evidence points to the presence in them of some factor capable of inducing malignant mesoblastic tumours along the path of their absorption in addition to the benign gastric lesions already described.

SUMMARY.

1. Rats maintained on (*a*) adequate diet, (*b*) restricted diet (bread or potatoes and milk), to which were added lard heated to 220° C. or 350° C., developed multiple mesenteric sarcomata in 4 out of 71 animals surviving more than 300 days. These animals were not infested with *Cysticercus fasciolaris*, which is known to be associated with sarcoma in the liver of infested rats.

2. Some rats on the restricted diet also developed benign gastro-papillomatosis.

3. Among 11 rats infested with *Cysticercus fasciolaris* 3 developed spindle-cell sarcomata attributable to the parasite.

4. Reasons are given for thinking that multiple mesenteric sarcomata in uninfested rats fed with heated fat were induced by a potential carcinogen acting on the mesoblastic tissues encountered in the process of absorption.

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THE EFFECTS OF TEMPORARY OCCLUSION OF THE RENAL CIRCULATION IN THE RABBIT.

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THE experiments described were designed to show the effects of temporary occlusion of the renal circulation on the structure and functions of the kidney.

METHODS.

The rabbits were anaesthetized with ether. The right kidney was then removed, the incision sewn up, and a fresh incision made in the left loin and the left kidney delivered on to sterile lint ; a small clamp was applied to the whole renal pedicle and the kidney replaced in the abdomen and the wound temporarily closed. Anaesthesia was continued with nembutal. The clamp was removed after varying periods up to 120 minutes, under ether when necessary, and the wound closed. In this experiment 30 rabbits were used, and of these 23 survived the operation for over three days.

RESULTS.

Blood urea.

After recovery blood was taken at intervals for blood urea estimation. (We are indebted to the Courtauld Institute of Biochemistry for these results.) Fig. 1 gives the results of these estimations in 7 rabbits.

It will be seen that during the first few days there is a rapid rise in the blood urea to approximately 300 mg. per 100 c.c. After that there may be continued rapid rise in the blood urea with death of the animal or there may be slow recovery. No deciding factor has been found which will determine if the outcome is to be recovery or death. It does not seem entirely to depend on the duration of the obstruction within the limits of the experiment, i.e. 60–120 minutes. In Rabbit B (Fig. 1) the renal circulation was only obstructed for 90 minutes, whereas in Rabbit E it was obstructed for the full 120 minutes.

Blood pressure.

Blood-pressure determinations were made in these animals, some by the capsule method and some by means of Van Leersum's loops. In no case was any significant rise in blood pressure detected. During the operations anaesthesia might be expected to prevent any rise in blood pressure ; the absence of hypertension in the later stages seems difficult to explain, but has been observed by other workers (Prinzmetal, Lewis, Taggart, Wilkins and Drury, 1940).

Kidney changes.

Naked eye.—In those rabbits which were killed during the first few days of the experiment the kidneys appeared pale and swollen, with loss of differentiation between cortex and medulla. In those which survived longer the kidneys were enlarged as a result of compensatory hypertrophy, but otherwise appeared normal.

Microscopical.—In those rabbits killed between the 2nd and 4th day marked changes were seen. These appeared to be confined mainly to the first portion of the secreting tubules, which showed gross degenerative changes with complete absence of nuclei and replacement of tubular substance by amorphous debris. (Figs. 2 and 3).

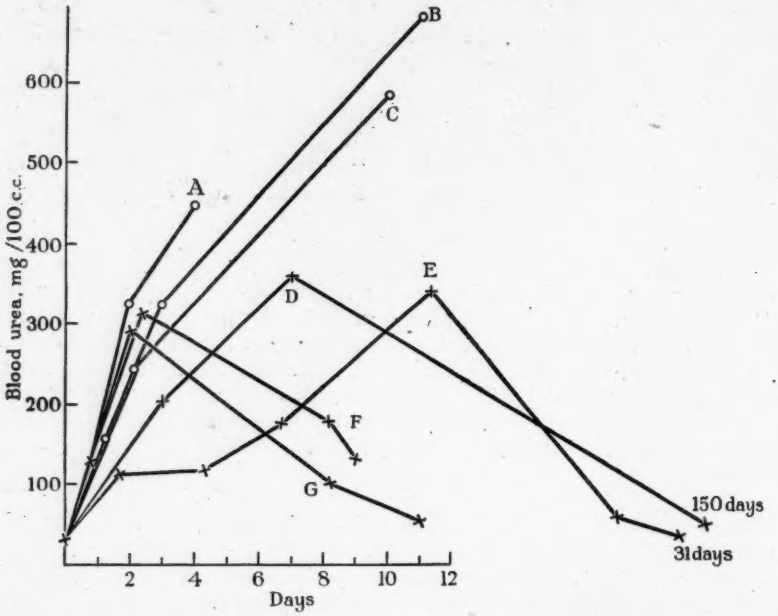
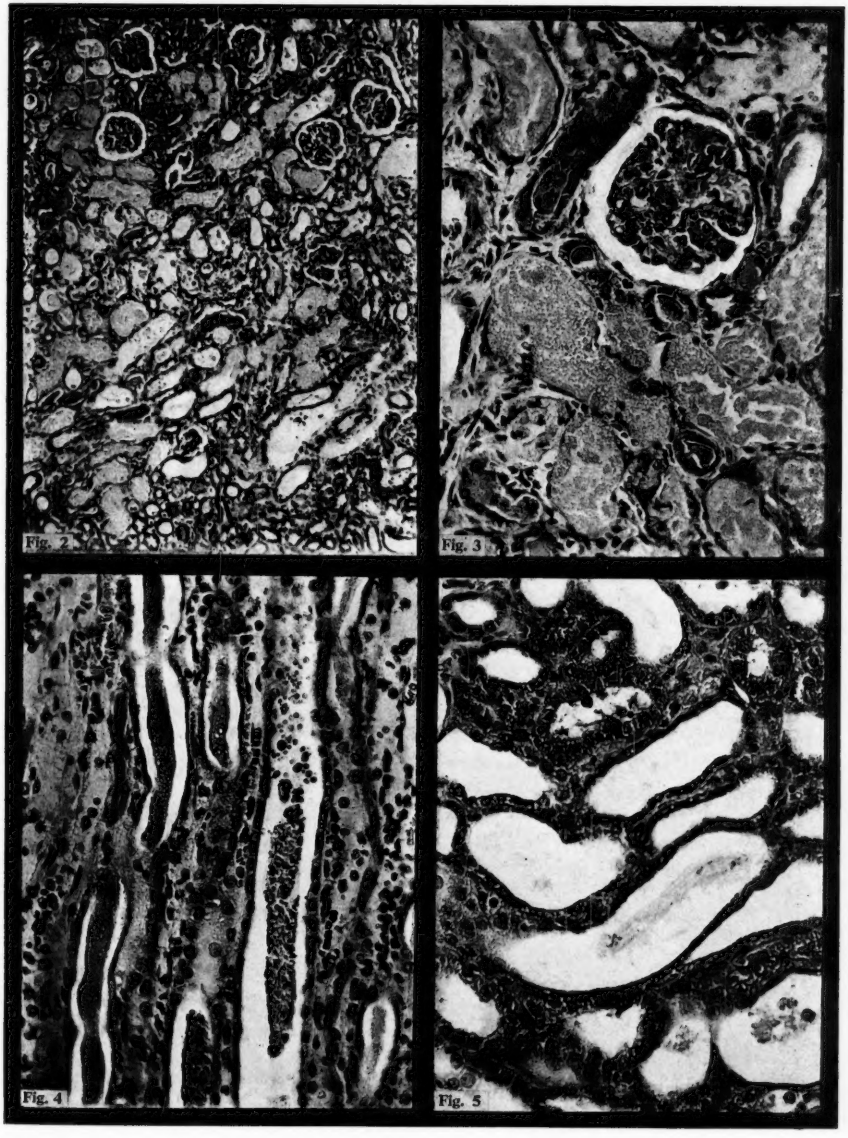


FIG. 1.—Blood urea after occlusion of renal circulation. Time of occlusion in minutes, A, C and E = 120; B and D = 90; F = 105.

Little fatty material could be detected on frozen section. The glomeruli appeared unaffected, but the distal parts of the secreting tubules and the collecting tubules showed numerous granular and hyaline casts (Fig. 4). Animals killed about the 10th day showed a histological picture which varied with the severity of the effects of the obstruction. In those animals in which the blood urea was rising progressively the changes were characterized by thinning of the epithelium of the proximal portions of the secreting tubules (Fig. 5). In those animals in which the blood urea was falling there was evidence of regeneration of tubular epithelium. Animals killed at a later date, i.e. 21 days and after, showed apparently normal kidney structure.

DISCUSSION.

In many conditions fatal anuria has been considered to be the result of obstruction of the kidney tubules by various substances; for example, the renal failure of crush injury is attributed to obstruction of the renal tubules by myohaemoglobin, and the



FIGS. 2, 3 and 4.—Showing extreme degeneration in secreting tubules and casts in collecting tubules (Rabbit A, Fig. 1).
FIG. 5.—Later stage of tubular change (Rabbit C, Fig. 1).

failure in sulphonamide poisoning to obstruction by acetyl sulphonamide. Damage to tubule cells has been considered to be the result of this obstruction, but it is possible that tubular damage is the primary lesion and intra-tubular deposition secondary to the oliguria. Our experiments were particularly designed to determine whether interruption of renal blood flow could produce tubular damage of the type seen following severe crushing injuries.

Complete renal ischaemia for two hours in rabbits produces pathological changes in the kidney very similar to those seen in human cases of " crush kidney " syndrome. Thus there is a possibility that the kidney lesion in cases of crush injury might be due to renal ischaemia, possibly during the period of limb compression, but more probably during the shock period which follows release from crushing; complete renal ischaemia is, of course, most improbable, but the possibility of severe partial ischaemia is suggested by an observation of Black, Powell and Smith (1941), who noted in a case of haematemesis a reduction of perabrodil clearance (taken as a measure of renal blood flow) to about 1/10 of normal. A prolonged reduction of blood flow of this degree might produce kidney damage of the type seen with complete ischaemia in rabbits.

It has generally been assumed that " crush kidney " lesions are not of ischaemic origin, since the blood pressure in the brachial artery is often not much reduced, but it must be remembered that renal blood flow can vary independently of the general blood pressure. In the case quoted by Black *et al.*, when the blood flow through the kidney was probably reduced to 1/10 of normal the blood pressure was 95/50. Mason and Mann (1931) recorded a diminution of kidney volume, presumably due to renal vasoconstriction, in anaesthetized and unanaesthetized dogs when haemoglobin solutions were injected intravenously; this vasoconstriction was not accompanied by any change of carotid blood pressure.

Thus one cannot exclude the possibility that primary renal damage may be responsible for a number of conditions which have been hitherto regarded as the result of intra-tubular obstruction by various substances; the presence of the various substances in the tubules may be an effect and not the cause of the kidney failure.

SUMMARY AND CONCLUSIONS.

Complete obstruction of the renal circulation in rabbits for 60–120 minutes results in extensive damage to the upper portions of the secreting tubules, accompanied by a rise in blood urea up to 300 mg. per 100 c.c. during the first 3–4 days after obstruction.

After this recovery may occur, or the condition may go on to death in uraemia with blood urea up to 650 mg. per 100 c.c.

The histological changes in the kidney show similarity to those seen in human cases of the " crush syndrome."

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VIABILITY OF *TREPONEMA PALLIDUM* IN STORED PLASMA.

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THE recent increase in the use of stored blood, plasma and serum for transfusion has aroused considerable interest in the question of how long *Treponema pallidum* remains infective in such fluids at low temperatures. During the past two years three attempts have been made to answer this question by preparing suspensions of rabbit chancre in rabbit plasma and testing their virulence after storage at 5° C. for various periods. In the first experiment tests were made at intervals up to 48 hours and all samples were virulent. In the second experiment, of which the details are given below, tests were made up to 6 days and, again, virulence was present up to the full period of testing. In the third experiment infected plasma tested for virulence after 6, 10 and 21 days failed to produce lesions in any of the inoculated rabbits. Since all three experiments were performed in a similar manner only the protocol of Experiment 2 will be given here.

EXPERIMENTAL.

Protocol of Experiment 2.—16 c.c. of blood was withdrawn from the ear vein of a normal rabbit into a 20 c.c. syringe containing 4·0 c.c. of 3 per cent. sodium citrate. The citrated blood was then centrifuged and 15 c.c. of plasma was separated. A 7-week-old rabbit testicular chancre (Levaditi's virus Truffi) weighing about 1·5 g. was then finely minced with scissors and suspended in the plasma. The suspension, which contained about 10 free spirochaetes per field in a dark-ground preparation, was then distributed in three small tubes (3 in. × ½ in.). The tubes, which were about three-quarters full, were stoppered with rubber bungs and placed in a refrigerator at 5° C. Virulence tests were made from successive tubes at 3, 4 and 6 days. At each test the tube was centrifuged at 2000 r.p.m. for 5 minutes and 2·0 c.c. of the supernatant plasma was separated. Two rabbits were then inoculated with grafts picked from the centrifuge deposit in the right testicle and with 1·0 c.c. of the supernatant plasma in the left testicle. The inoculated rabbits were then examined weekly for the presence of syphilitic lesions. When these were detected, samples were aspirated and examined by dark-ground illumination. All the lesions showed the presence of numerous *Tr. pallida*. From the results summarized in Table I it can be concluded that under the conditions of this experiment syphilitic plasma can remain infective for at least 6 days.

DISCUSSION.

The period of survival found here at refrigerator temperature is longer than that found by other workers. Bloch (1941), using a smaller inoculum of syphilitic tissue from rabbit chancre in whole rabbit blood, reported that *Tr. pallidum* retained its virulence at 3° to 5° C. for 3 days, but not for 4 days. Turner and Diseker (1941),

TABLE I.—*Virulence of* Treponema pallidum *during Storage in Plasma at 5° C.*

Number of days' storage at 5° C.	Number of injected rabbit.	Number of days to detection of lesion after inoculation of—	
		Tissue deposit.	Supernatant plasma.
3	153	30	42
	154	35	Negative
4	155	41	49
	156	41	56
6	157	46	Negative
	158	39	39

and Kolmer and Rule (1942), using still smaller infecting inocula in human stored blood, found that the spirochaete remained virulent for rabbits at refrigerator temperature for less than three days. The influence of the infecting dose is also evident in Table I, where it can be seen that lesions from the inoculation of the more heavily infected tissue deposit are more frequent, and usually appear earlier than those from the inoculation of the supernatant plasma. Another factor which may account for these differences in survival time is the presence of tissue which, as Bloch (1941) suggests, may have a favourable influence on maintaining virulence. It would therefore appear that, although syphilitic plasma may remain infective at 5° C. for 6 days in favourable conditions, the probability is that plasma derived from human syphilitic blood stored at refrigerator temperature would lose its virulence well within that period. On the other hand, when spirochaetes are maintained in the frozen state at — 78° C. they may remain virulent for at least 3 years (Turner and Fleming, 1939), but at — 20° C. virulence disappears within 2 months (Turner, 1938). To explain the greater lability of spirochaetes at temperatures nearer freezing point, Turner and Brayton (1939) suggest that at these temperatures injury is brought about mainly by changes incident to cell metabolism or to proteolytic or other enzymes, while at — 78° C. these biological activities are suspended.

In considering how far these results bear on the question of transfusion syphilis allowance must be made for the experimental conditions. In the first place, the numbers of spirochaetes in the infected plasma were many times greater than those encountered in human syphilitic blood. Secondly, the infected plasma was injected, not into the blood stream, but into a sensitive tissue, thus largely precluding the operation of the natural defences of the host. The risk of transmitting syphilis by blood transfusion would not then be so great as these experiments would appear to indicate. This appears to be borne out to some extent by the reports on transfusion syphilis. Although a considerable number of cases of syphilis following direct blood transfusion have been reported, none appears to have followed the transfusion of stored blood, serum or plasma. Even with direct transfusion the risk appears to be small in this country, where only one case has so far been reported (McCluskie, 1939). Another notable feature in the reports of transfusion syphilis is the high proportion of cases in which the blood donor is a near relative of the recipient. This alone would lead one to suspect that, at least in some cases, the infection was determined by the social conditions of the patient and was not transmitted by transfusion. It is also remarkable that there has been no increase in the reports of transfusion syphilis coincident with the great increase in the practice of transfusion in recent years.

The available evidence would therefore seem to indicate that in populations where the general incidence of syphilis is low and where reasonable precautions are taken there is little danger of transmitting syphilis by transfusion. The use of stored transfusion fluids would further diminish this risk, since 6 days would appear to be the

maximum time that *Tr. pallidum* can remain virulent in the most favourable experimental conditions. The use of serum or plasma would be an added safeguard because their preparation involves not only mixing but also clotting and spinning, whereby a large proportion of spirochaetes in an infected blood would be separated. On the whole it would appear that, although *Tr. pallidum* has considerable powers of survival in stored plasma, the risk of transmission of syphilis by stored blood or blood products is small.

SUMMARY.

Rabbit plasma infected with a rabbit strain of *Tr. pallidum* and stored at 5° C. remained infective for 6 days.

Although *Tr. pallidum* has considerable powers of survival in stored plasma, reasons are given for suggesting that the risk of the transmission of syphilis by transfusion with stored blood or blood products is small.

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NEUTRALIZATION OF SOME PLANT VIRUSES BY RABBIT SERA.

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SINCE Mulvania (1926) showed that the addition of normal rabbit serum to tobacco mosaic virus caused a decrease in infectivity, various workers have confirmed this, but found that the serum of rabbits immunized with the virus has a still greater effect. Chester (1934) has made the most detailed studies of this phenomenon. He claims that the effect of virus antisera differs qualitatively as well as quantitatively from that of normal antisera, and that heterologous virus-antisera behave like normal sera, while sera prepared against serologically related virus strains behave like homologous sera.

With many animal viruses and bacteriophages, neutralization of infectivity is the most sensitive serological test, and can be used for demonstrating serological relationships where the precipitin test cannot be applied. This work was started to see if the virus neutralization test could be applied to determine relationships between the viruses causing tobacco necrosis. Bawden (1941) and Bawden and Pirie (1942) have shown that this disease can be caused by a number of viruses, some of which precipitate with each other's antisera, whereas others do not. It seemed possible that neutralization tests might show relationships between these viruses which cause identical symptoms, even though precipitin tests do not. However, it soon became obvious

that neutralization tests with plant viruses could not be interpreted as simply as earlier work had suggested, and that the problem is complicated by many variable factors. The work was therefore extended to other viruses and sera to get further information on the mechanism of neutralization.

Material and Methods.

The viruses used were tobacco mosaic virus (T.M.V.), tomato bushy stunt virus (B.S.V.), and the two cultures of tobacco necrosis viruses called by Bawden and Pirie (1942) tobacco II (T.N.II) and potato (T.N.P.). Thirty different sera were used : Ten normal rabbit sera, seven against T.M.V. (two were against heat denatured virus), four against B.S.V. (one was against heat denatured virus), five against the two tobacco necrosis viruses, two against the aucuba mosaic strain of tobacco mosaic virus, one against the enation mosaic strain of tobacco mosaic virus, and one against human albumin. The sera were prepared by Dr. A. Kleczkowski by three to six intravenous injections, and were kept at 2° C. with 0·5 per cent. phenol or some thymol, unless otherwise specified.

Infectivity tests were made either by the opposite half leaf method or by a Latin square arrangement. At least six replications were made in each test. Infectivity tests with tobacco necrosis viruses were made on beans (*Phaseolus vulgaris* var. Canadian Wonder) and with B.S.V. and T.M.V. on *Nicotiana glutinosa*. Purified preparations of B.S.V. and T.M.V. were always used, while tests with the tobacco necrosis viruses were made with infective sap clarified by freezing and centrifuging. In all tests an equal volume of virus solution and either serum or saline were mixed thoroughly at least 30 minutes before being rubbed on the test plants.

The Neutralizing Power of Sera.

In an attempt to establish a base line for the unspecific neutralization by normal sera, blood samples were taken from five normal rabbits of the same litter. The sera were tested the day after preparation, and were compared with a stored sample of normal serum and antisera to T.M.V. and B.S.V. for their ability to neutralize T.M.V. The results in Table I show that there are differences between the behaviour of the individual sera of the same age, but these are insignificant compared with those between the sera of different ages. Indeed the effect of all the fresh normal sera is greater than that of the stored homologous antiserum ; it will be noticed that antiserum to B.S.V. is also more effective than the homologous serum.

A possible explanation of the difference between new and old serum was the presence

TABLE I.—*Neutralizing Effect of Sera of Different Ages on Tobacco Mosaic Virus.*

Serum.	Age of serum.	Total number of lesions.
Fresh normal serum A	1 day	20
,, ,, ,, B	,,	39
,, ,, ,, C	,,	18
,, ,, ,, D	,,	22
,, ,, ,, E	,,	13
Old normal serum	15 months	234
B.S.V. antiserum	3 ,,	35
T.M.V. ,,	7 ,,	54
Saline control	..	350

1 ml. of sera at 1/20 or 1 ml. of saline was mixed with 1 ml. of 0·003 per cent. T.M.V.

of active complement in the former, but tests with heated sera showed that this was not the correct one. Two freshly-prepared normal sera were tested before and after heating at a dilution of 1/10 for 20 minutes at 56° C.; T.M.V. mixed with saline gave 338 lesions, while the mixtures with the two unheated sera gave 3 and 7, and the mixtures with the heated samples gave 4 and 5 respectively. Similarly, heating old sera to this extent also had no effect on its neutralizing power. The fact that heating does not duplicate the effect of ageing suggests that the loss of neutralizing power during storage may result from some enzymatic action, which is inhibited by heating to 56° C. That enzymatic action might be the cause was also suggested by the rates at which the neutralizing power fell off in different conditions of storage. At 2° C. one sample of normal serum, tested at a dilution of 1/20, reduced the number of lesions with tobacco mosaic virus by 89 per cent. after 1 day, 79 per cent. after 7 days, 57 per cent. after 28 days and 42 per cent. after 62 days, whereas a sample kept frozen retained its full neutralizing power when tested after 80 days.

The neutralizing power of freshly prepared normal sera is so great that any specific effects of fresh homologous sera are small by comparison, and these are shown at all easily only after sera have been stored for some months, when normal serum at 1/20 reduces the infectivity of 0·003 per cent. solutions of T.M.V. by about 50 per cent. Even then, however, a comparison of the inhibitory effect of normal serum and an antiserum is often insufficient to act as a test of a specific serological relationship, for heterologous antisera are usually better neutralizers than are normal sera. This phenomenon is clearly shown in Table II, where it will be seen that both T.M.V. and B.S.V. are more affected by any of the antisera used than by normal serum. With B.S.V. there is evidence of a further specific effect of the homologous serum, but there is none with T.M.V. Thus if a greater neutralization power than normal serum is used to indicate a specific reaction, it could be concluded that not only are the three viruses related to one another, but to human albumin also. From the results of a large number of similar tests with many viruses and sera, it seems that the introduction of an antigen of any kind into a rabbit increases the ability of its serum to neutralize the infectivity of plant viruses.

This unspecific effect of a heterologous antiserum applies equally against all the viruses used; for example, in Table II, although B.S.V. is affected more than T.M.V. by all the sera, the neutralizing power of the heterologous sera falls in the same order for both B.S.V. and T.M.V. Thus, in attempting to use neutralization as a test of virus relationship, it is necessary to prepare sera separately against the viruses, store them under similar conditions and do reciprocal tests, using each virus mixed with each serum. Then, except for exceptionally strong inhibitory sera, such as those to T.N.P. and B.S.V. (see Table I), specific reactions can be demonstrated, although they

TABLE II.—*Neutralizing Effect of Different Sera on Tobacco Mosaic Virus and Bushy Stunt Virus.*

Serum.	Number of lesions as percentage of control.	
	T.M.V.	B.S.V.
T.N.P. antiserum	17	6
Human albumin antiserum	28	17
T.M.V. antiserum	20	14
B.S.V. ,,	8	0·6
Normal rabbit serum	39	20
Saline control	100	100

1 ml. of sera at dil. 1/20 or 1 ml. of saline was mixed with 1 ml. of T.M.V. 0·003 per cent. or 1 ml. of B.S.V. 0·01 per cent.

are usually small. This is shown in Table III, where comparison of the effects of different sera on various viruses are given ; it will be seen that the specific reductions are restricted to antisera which also cause precipitation.

Thus the neutralization tests confirm the fact that tobacco necrosis is a disease caused by serologically distinct viruses, and suggest the same relationships as precipitin tests.

TABLE III.—*Relation between Precipitin Reaction and Neutralization.*

Virus.	Antisera.	Precipitation.	Number of lesions as percentage of saline control.
T.N. II	T.N. II	+	4%
	T.N.P.	—	16%
T.N.P.	T.N.P.	+	7%
	T.N. II	—	26%
	B.S.V.	—	41%
B.S.V.	B.S.V.	+	5%
	T.N.P.	—	32%
	T.M.V.	—	8%
T.M.V.	T.M.V.	+	7%
	B.S.V.	—	21%
Aucuba mosaic virus	Enation mosaic virus	+	7%
	Aucuba ,, ,,	+	4%
	T.M.V.	+	9%
	Aucuba mosaic virus	+	4%
Enation mosaic virus	Enation ,, ,,	+	10%
	T.M.V.	+	10%
	Aucuba mosaic virus	+	9%
	Enation ,, ,,	+	7%

Precipitating Antibodies and Virus Neutralization.

As all precipitating antisera were better neutralizers on infectivity than normal sera and the two best, against T.N.P. and B.S.V. respectively, had high precipitating titres, it seemed possible that the content of precipitating antibodies might determine the ability to neutralize infectivity. However, experiments of several different kinds have failed to confirm this. The ability of many different T.M.V. antisera, prepared at different times and with titres ranging from 1/200 to 1/1500, to neutralize the infectivity of the virus bore no relationship to their precipitating power, those with lower titres often being more effective than those with higher. This was tested more carefully by deliberately preparing sera against T.M.V. and B.S.V. with widely different precipitin titres. Bawden and Kleczkowski (1942) have shown that these viruses retain some antigenicity after being denatured by heat, but that they then give sera with only low precipitation titres. Sera were therefore prepared separately, using rabbits from the same litter, against these viruses both in the active state and after denaturation by heating for 10 minutes at 95° C. Each rabbit received the same amount of virus. The sera were stored under identical conditions and tested for their precipitin titres and ability to neutralize infectivity. It will be seen from Table IV that the sera produced by injecting denatured viruses were as effective in neutralizing infectivity as the others, in spite of their much smaller content of precipitating antibodies.

To obtain more evidence on this point, experiments were made with sera from which

TABLE IV.—*Neutralizing Power of Antisera Prepared Against Active and Denatured Viruses.*

Antigen used for preparing sera.	Precipitin titre of sera.	Number of lesions with sera at dilutions— 1/20.	1/200.
		T.M.V.	
Heated T.M.V.	1/20	21	109
Unheated T.M.V.	1/400	51	110
	Saline control	220	
		B.S.V.	
Heated B.S.V.	1/1	2	75
Unheated B.S.V.	1/160	3	87
	Saline control	324	

1 ml. of antiserum at 1/20 or 1/200, or 1 ml. of saline, was mixed with 1 ml. of 0·003 per cent. of T.M.V. or 0·03 per cent. B.S.V.

the precipitating antibodies were removed. First tests were made to find the effect of the unspecific neutralization. 1 ml. lots of T.M.V. antisera and of normal sera diluted 1/10 were mixed with 1 ml. of 0·27 per cent. T.M.V. and allowed to stand for 24 hours. They were then centrifuged and the supernatant fluids compared with unabsorbed serum for their ability to neutralize T.N.P. The saline control gave 905 lesions, the mixture with normal serum 414, and the absorbed and unabsorbed T.M.V. antisera gave 214 and 272 respectively. Similar results were also obtained with antiserum to human albumin, when tested against T.M.V. and T.N.P. Both viruses were affected as much by the absorbed as by the unabsorbed antiserum, and the removal of all precipitating antibodies apparently had no effect on the unspecific neutralizing power.

Other tests were made to see if the precipitating antibodies were responsible for the specific neutralization of homologous virus antigens. 0·2 per cent. T.M.V. were mixed with 2 per cent. formaldehyde for two days at room temperature and then dialysed. This treatment destroys infectivity without affecting the ability of the virus to be precipitated by its antiserum. The formolized virus was then used at antigen excess to absorb all the precipitating antibodies from T.M.V. antiserum, and the absorbed antiserum after centrifugation was compared for its ability to neutralize T.M.V. with the unabsorbed and normal serum at the same dilutions.

Table V shows that the removal of all the precipitating antibodies has had no effect on the specific neutralization of T.M.V. Thus, although it has been shown that specific neutralization of the viruses only occurs with sera that cause precipitation, yet the precipitating antibodies do not seem to play any appreciable part in the neutralization.

TABLE V.—*The Effect of Removal of Precipitating Antibodies on the Neutralization of Tobacco Mosaic Virus.*

Serum.	Total number of lesions (average from 5 experiments) with sera at dilutions— 1/1.	1/10.
Absorbed with formolized T.M.V.	55	186
Unabsorbed	53	164
Normal	197	280
Saline control	357	476

The test was done with 0·0003 per cent. T.M.V. mixed with equal volume of sera.

TABLE VI.—*Infectivity of Tobacco Mosaic Virus-Antiserum Precipitate.*

Preparations.	Total number of lesions at dilutions—		Percentage number of lesions at dilutions—	
	1/1.	1/10.	1/1.	1/10.
Virus-antiserum mixture . . .	50	43	5	16
Supernatant fluid	18	33	2	12
Precipitate resuspended in saline . .	410	181	47	70
Precipitate resuspended in antiserum at 1/20	43	78	4	30
Precipitate resuspended in normal serum at 1/20	163	119	18	46
Saline control	861	255	100	100

Description in text.

This fact is further emphasized by tests of the infectivity of virus-antiserum precipitates (Table VI). 1 ml. lots of 0·016 per cent. T.M.V. and T.M.V. antiserum at 1/20 were mixed, incubated for 2 hours at 50° C., left overnight at room temperature and centrifuged. The precipitates were resuspended in either 2 ml. of saline, 2 ml. of normal rabbit serum at 1/20 or 2 ml. of T.M.V. serum at 1/20, and their infectivity compared with the original virus-antiserum mixture, the supernatant fluid, and with a similarly incubated virus-saline mixture.

Similar results have also been obtained with B.S.V. In one, for example, the virus-antiserum mixture had only 16 per cent. of the infectivity of the saline control, whereas virus-antiserum precipitate resuspended in saline had 56 per cent. If the precipitates are washed with saline, their infectivity can be still further increased, suggesting that the precipitate has carried with it some substances responsible for neutralization. Such resuspended precipitates, however, have never been as infective as the original virus. This may mean that the precipitating antibodies do have some neutralizing effect, but it is equally likely that it is due to other substances absorbed on to the precipitate or to comparing the infectivity of soluble virus with insoluble aggregates. As such insoluble material has so much infectivity, however, it is clear that other substances than the precipitating antibodies are largely responsible for both the unspecific and specific neutralization effect of antisera.

Quantitative Differences between Homologous and Heterologous Sera.

Chester (1934) stated that the neutralization of viruses by normal sera or heterologous sera differed qualitatively as well as quantitatively from neutralization by homologous sera, and he suggested that the former acted by altering the susceptibility of the host plant and the latter acted directly on the virus. No evidence for this view has been obtained in the experiments described here, where the only differences found between different sera have been quantitative.

The results in Table VI show that diluting has resulted in some increase in infectivity of the virus-serum mixtures as compared with the saline control. This is a constant phenomenon, and occurs with homologous and heterologous sera. A more detailed test of this type is shown in Table VII, where the results of diluting mixtures of T.M.V. with four different sera and saline are given. Only quantitative differences are seen, for the mixtures with T.M.V. and aucuba mosaic virus antisera merely need greater dilution than mixtures with B.S.V. antiserum or normal serum to regain their full infectivity.

Chester based his differentiation on two main factors. Firstly, he found that the

TABLE VII.—*Effect of Dilution on the Infectivity of Mixtures of Tobacco Mosaic with Different Sera.*

Serum.	Total number of lesions at dilutions—			
	1/1.	1/10.	1/100.	1/1000.
Against T.M.V.	17	12	9	5
,, aucuba mosaic	6	29	8	5
,, B.S.V.	38	86	32	9
Normal	227	166	40	8
Saline control	461	148	21	6

effect of homologous sera increased with time of contact with the virus, while other sera produced their maximum effect immediately on mixing ; and, secondly, he found that the effect of a constant amount of homologous antiserum produced increasing percentage loss of infectivity when mixed with decreasing amounts of virus, while the heterologous sera produced a constant percentage loss. Neither of these effects has been obtained in this work.

Tests have been made in which T.M.V. has been mixed with various sera and the mixtures tested for their infectivity after various periods of incubation ; with no serum was incubation found to decrease infectivity, and homologous and other sera all produced their maximum effects immediately on mixing. Similarly, when homologous and other sera were tested at a constant dilution against various dilutions of virus, only quantitative differences were obtained. Table VIII shows one such test in which T.M.V. at different dilutions was mixed with equal volumes of T.M.V. antiserum or normal serum at 1/50, and it will be seen that the reduction in infectivity relative to the saline control increases with both sera as the dilution of virus increases.

It is difficult to gain direct evidence as to whether a substance is reducing infectivity by acting on the host plant or on the virus. Much more reduction is obtained if the viruses and sera are mixed *in vitro* and the mixture rubbed on to the plant than if the constituents of the mixture are inoculated separately. In one experiment with T.M.V. and its homologous antiserum, for example, the mixture gave 9 per cent. of the lesions formed by the saline control, serum followed by virus gave 53 per cent., and virus followed by serum 68 per cent. Similar results differing only quantitatively from these were obtained with other viruses and either their homologous or other sera. An effect on the susceptibility of the host plant is not excluded by these results, but no qualitative differences have been found between the action of homologous and other sera ; it seems most likely that the neutralization of viruses, both specific and unspecific,

TABLE VIII.—*Neutralization Effect of Constant Amount of Sera on Various Dilutions of Tobacco Mosaic Virus.*

Dilution of T.M.V.	Number of lesions as percentage of saline control.*	
	T.M.V. antiserum. 1/50.	Normal serum. 1/50.
10^{-3}	23	53
10^{-4}	10	18
10^{-5}	7	11
10^{-6}	0	21
10^{-7}	0	0

* The saline control gave 668, 367, 118, 33 and 11 at 10^{-3}, $^{-4}$, $^{-5}$, $^{-6}$ and 10^{-7} respectively.

results from the virus combining with substances in the sera to give non-infective complexes, which, however, easily dissociate to liberate infective virus.

SUMMARY.

The unspecific neutralization of plant viruses by normal and heterologous sera was so large that the additional specific effect of homologous antisera was small in comparison. This specific neutralization could be used for demonstrating serological relationships only if sera of the same age and stored similarly were compared; the relationships indicated were the same as those indicated by precipitin tests.

Unless sera were kept frozen their unspecific neutralizing power fell rapidly on storing. All heterologous antisera reduced infectivity more than normal sera, stored comparably.

Precipitating antibodies did not appear to be responsible for neutralization. No correlation was found between precipitin titre and neutralizing power, and removal of precipitins did not affect neutralization power. Only quantitative differences were found in behaviour between homologous and other sera; the infectivity of all virus-serum mixtures was regained by dilution.

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TYPE RELATIONSHIPS AMONGST GROUP A STREPTOCOCCI.

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GRIFFITH (1935) described a serological technique by which he was able to classify the majority of streptococci that he isolated from acute epidemic human infections. He described two main difficulties inherent in his method: First, it was often difficult to obtain stable bacterial suspensions suitable for slide agglutination tests; second, it was common for crude antisera to agglutinate streptococci of heterologous type.

In attempting to overcome the first difficulty different methods of culture may be used varying the constituents of the nutrient broth or the temperature at which the organisms are grown. Griffith described several different broth media, each of which was sometimes successful when the others failed to yield stable suspensions. Shibley (1924) made the observation, later confirmed by Colebrook (1935), that streptococci which yield granular suspensions when grown at 37° C. often grow uniformly if incubated at room temperature. When these methods fail the streptococci may be treated by special methods after growth—for example, by tryptic digestion (Allison) or by suspension in normal serum (Edward, 1941).

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The difficulty of non-specific agglutination may be overcome to some extent by removing the non-specific agglutinins from the crude sera by absorption with streptococci of heterologous type. In his earlier work in this field Griffith absorbed the crude " typing " sera with a mixture of heterologous types, but latterly he had preferred to use a single strain. This, a variant of a Type 3 streptococcus, was found to be rich in non-specific antigens but to contain little or no type-specific antigen. Thus it was strongly agglutinated by almost all crude typing sera, but did not react with Type 3 antisera, whose non-specific agglutinins had been absorbed by streptococci of heterologous type (Griffith, 1935).

Despite these modifications of technique the identification of unknown strains is still complicated by the cross reactions which constantly occur between certain types and heterologous antisera.

Recently Lancefield (1940*a*) has shown that some Group A streptococci contain two or more antigens which she calls M and T substances : both types of antigen may participate in the agglutination of streptococci by homologous sera absorbed by Griffith's method. She suggests, moreover, that the degree of specificity of these substances may vary in different streptococcal types, resulting in some of the anomalous cross reactions constantly encountered in the identification of Group A strains.

In the present inquiry certain cross-reacting types have been subjected to a form of crude antigenic analysis with a view to determining the nature of the common and type-specific antigens in terms of the M and T factors postulated by Lancefield. It must be stated at the outset, however, that although Lancefield has characterized the M antigens as possessed of certain definite properties, " T substance " is merely a term applied to a hypothetical group of antigens. It is by no means certain, therefore, that the factors referred to as T substances in this work and those so described by Lancefield are one and the same.

THE RELATIONSHIP BETWEEN TYPES 4 AND 24.

In investigating the relationship between these two types the strains Wheatley (Type 4) and G25 (Type 24) were used exclusively in the preliminary experiments, the results of which were subsequently tested with other strains belonging to these two types.

Technique.

Agglutinating suspensions were grown in heart digest broth incubated for 18 hours at 37° C. or for several days at 22° C. In the 4/24 investigation broth without glucose was used, but in subsequent experiments with Types 1, 14 and R491, 0·2 per cent. of glucose was added. *Antisera* were prepared by inoculating rabbits with formolized cultures of G25 and Wheatley. Before use non-specific agglutinins were removed by absorption with a non-specific Type 3 variant. Further *agglutinin absorption* tests were then carried out using a slightly modified form of the technique recommended by Griffith (1927). 0·5 c.c. of a 1 in 5 dilution of serum in saline was mixed with successive doses of the centrifuged deposit of 100 c.c. of an 18-hour heart digest broth culture grown at 37° C. Each 100 c.c. of culture represented an absorbing dose of approximately 10,000 million cocci. The sera and cocci were allowed to remain in contact for 1 hour at room temperature. The mixture was then centrifuged and the serum tested for residual agglutinins with suspensions of Wheatley and G25 grown either at 37° C. or 22° C., in some instances digested with trypsin for 18 hours at 37° C. or heated to 100° C. for 15 minutes. Each sample of serum was repeatedly absorbed until after two successive absorptions the antibody titre remained constant. *Antibody titrations* were carried out by the slide agglutination method (Griffith, 1927). Satisfactory end-points were readily obtained, but the results were generally confirmed by water-bath agglutination tests.

"*M* " *extracts* were prepared from the cultures in the manner described by Lancefield (1928) and tested against the variously absorbed sera by the ring test. In *precipitin absorption* tests the freshly prepared M substance from 100 c.c. of an 18-hour broth culture grown at 37° C. was added to 0·25 c.c. of serum. The mixture was incubated for 1 hour at 37° C. and then left in the refrigerator until the following day, when it

was centrifuged and the absorbed serum tested for residual antibodies both by precipitation with M extracts and agglutination as in the agglutinin absorption experiments. The method of optimal proportions was not used because sera could not be obtained sufficiently strong in precipitins to stand dilution.

Comparison by agglutination of cultures grown at 37° C. and 22° C.

It will be seen from the results of the agglutination tests shown in Table I that strain G25 (Type 24) appears to exist in at least two phases, one being found in cultures grown at 37° C. which are type-specific and agglutinated only by homologous serum, the other in cultures grown at 22° C. which are agglutinated by both Type 24 and Type 4 sera. Wheatley (Type 4) is agglutinated by both Type 4 and Type 24 sera, irrespective of whether it is grown at 37° C. or 22° C.

TABLE I.—*Reactions of G25 (Type 24) and Wheatley (Type 4) with Absorbed Sera.*

	Serum.				
		G25 (Type 24) absorbed with Type 3 non-specific and—			
*Agglutinating suspensions and "M" extracts.	G25 absorbed with Type 3 non-specific.	G25 (37° C.)	G 25 (22° C.)	Wheatley (37° C.).	G25 "M" extract.	Wheatley (Type 4) absorbed with Type 3 non-specific.
G25 (37° C.)	+	−	−	+	−	−
,, (22° C.)	++	++	−	++	++	++
,, (37° C.) "trypsinized"	−	−	−	−	−	−
,, (22° C.)	++	++	−	−	++	++
,, (37° C.) boiled	+	−	−	+	−	−
,, (22° C.) ,,	+	−	−	+	−	−
G25 M extract	+	−	−	+	−	−
Wheatley (37° C.)	++	++	−	−	++	++
,, (22° C.)	++	++	−	−	++	++
,, (37° C.) "tryp."	++	++	−	−	++	++
,, (22° C.) "tryp."	++	++	−	−	++	++
,, (37° C.) boiled	−	−	−	−	−	−
,, (22° C.) boiled	−	−	−	−	−	−
,, "M" extract	−	−	−	−	−	−

* Agglutinating suspensions were grown in heart digest broth either at 37° C. or 22° C. Some were either digested with trypsin or heated to 100° C. after growth.

Agglutinin absorption experiments with cultures grown at 37° C. and 22° C.

G25 antiserum was absorbed with a type-specific culture of G25 grown at 37° C.; the type-specific antibodies were removed, but the absorbed serum still agglutinated Wheatley to the same titre as before absorption. Another sample of G25 serum was absorbed with a culture of Wheatley grown at 37° C. After absorption the serum no longer agglutinated Wheatley suspensions, but agglutinated type-specific G25 suspensions to the same titre as before absorption. Both absorbed sera still agglutinated 22° C. cultures of G25, but absorption of a third sample of G25 serum with such a culture removed all the agglutinins for both G25 and Wheatley suspensions.

From the results of these experiments (shown in Table II) it appears that G25 serum contains two different antibodies in addition to the non-specific streptococcal antibodies absorbed by non-specific Type 3. One is responsible for the type-specific agglutination of Type 24 cultures, the other for the agglutination of Type 4 suspensions. Of the corresponding antigens the first is dominant in G25 cultures grown at 37° C.,

the second in Wheatley cultures grown at 37° or 22° C. Both antigens are exposed in the G25 cultures grown at 22° C.

TABLE II.—*Reactions of G25 (Type 24) and Wheatley (Type 4) with Absorbed Sera, showing Titres of Residual Agglutinins.*

	Serum.							
		G25 (Type 24) absorbed with Type 3 non-specific and—				Wheatley (Type 4) absorbed with—	
Agglutinating suspension.	G25 absorbed with Type 3 non-specific.	G25 (37° C.).	G25 (22° C.).	G25 "M" extract.	Wheatley (37° C.).	Type 3 non-specific.	G25 (22° C.).
G25 (37° C.)	120	—	—	—	120	..	..
G25 (22° C.)	..	..	..	..	..	480	30
Wheatley (37° C.)	480	480	—	*240	—	480	30

The figures shown in the serum columns represent the reciprocals of the titres to which the suspensions were agglutinated on the slide.

* A different G25 serum was used for the absorption by "M" extract from that used in the other absorptions.

It will be seen from Table II that absorption experiments failed to produce evidence of type-specific agglutinins in the Wheatley antiserum. Absorption of this serum with six successive doses of a G25 22° C. culture reduced *pari passu* the titres for both G25 and Wheatley from 1 : 480 to 1 : 30.

Precipitation experiments with M extracts from G25 and Wheatley.

It will be seen from Table I that M extracts prepared from G25 were precipitated only by Type 24 serum. Absorption of this serum with such an extract removed not only the corresponding precipitins, but also the type-specific agglutinins. Lancefield (1940*a*) has shown that M substance is type-specific in certain strains, including some belonging to Types 1, 3, 6 and 14. It seems probable, therefore, that the type-specific agglutination of G25 depends on its possessing M substance, which participates in both agglutination and precipitation reactions.

Effect of heat on 37° C. and 22° C. cultures.

Suspensions of G25 and Wheatley were heated to 100° C. for 15 minutes in a water-bath. By this treatment they were usually found to be aggregated into coarse floccules, which were easily broken up by vigorous shaking and thereafter yielded stable suspensions suitable for agglutination tests.

G25 specific suspensions were apparently unaltered by this degree of heating as judged by agglutination tests shown in Table I; 22° C. cultures of G25, on the other hand, were no longer agglutinated by Type 4 serum, but still reacted with homologous serum and to the same titre as did the 37° C. type-specific cultures.

Wheatley suspensions were completely inactivated by heating to 100° C., and thereafter no longer reacted with either Type 4 or Type 24 sera.

Effect of tryptic digestion on 37° C. and 22° C. cultures.

Two drops of pancreatic extract were added to the deposit of 10 c.c. of a broth culture at pH 8·5 to 9·0. The suspension was tested by slide agglutination after

digestion had been allowed to proceed for periods varying from 30 minutes to 18 hours at 37° C.

Type-specific suspensions of G25 were agglutinated by neither Type 24 nor Type 4 serum after two hours' digestion. 22° C. cultures of G25 were no longer agglutinated by type-specific homologous serum (Type 24 serum absorbed with Type 4 cocci), but continued to be strongly agglutinated by sera containing antibody common to both types, i.e. Wheatley and G25 sera.

Agglutinating suspensions of Type 4 cocci were unaffected by tryptic digestion.

The investigation of Type 4 and 24 cultures other than Wheatley and G25.

Type 24 is apparently uncommon in England. Dr. Griffith left in his collection of type strains three belonging to this type. One of these was G25 described above. Of the other two, G54 shared the characteristics of G25 and was probably isolated from the same outbreak. The third strain, Turton, appears to have changed during subculture; it is agglutinated by Type 4 sera, but no longer reacts with Type 24 specific sera. An antiserum made with this strain when it was first isolated behaves like the G25 and G54 sera, so that originally the Turton strain presumably contained both the antigen specific to Type 24 and that common to Types 4 and 24. During the last two years I have encountered only one outbreak of infection due to Type 24 streptococci. The responsible organism, 1364, reacts only with Type 24 serum and fails to react with Type 4 serum, even when grown at 22° C. Its type-specific antigen is inactivated by tryptic digestion, but withstands a temperature of 100° C. This streptococcus, therefore, appears to possess the Type 24 specific M antigen, but to lack the Type 4 antigen. This supposition was confirmed by immunizing a rabbit with strain 1364. After a full course of inoculations the serum agglutinated or precipitated Type 24 cocci or their M extracts, but did not react with any Type 4 organisms tested.

It is the general experience that Type 4 is a common cause of streptococcal infection in this country. Of the numerous strains of this type that I have examined all corresponded in their properties with Wheatley, i.e. they were agglutinated by both Type 4 and 24 sera, continued to react in this way after prolonged tryptic digestion, but ceased to react after being heated to 100° C.

Summary and Conclusions.

If Wheatley and G25 are representative of most Type 4 and Type 24 streptococci, it seems that two antigens are responsible for the agglutination of these types by absorbed sera. One is specific to Type 24, the other is shared by both types. The first corresponds to the M substance described by Lancefield as type-specific in Types 1, 3, 6 and 14. The other may correspond to the T antigens recently described by her as being responsible for the agglutination by absorbed sera of strains belonging to those types, but lacking the M substance.

Using this nomenclature, Type 24 strains, G25 and G54, possess M and T antigens. The M antigen may be dominant in cultures grown at 37° C. This was so in the G25 cultures used in these tests, but was not invariable. It appeared to depend to some extent on the constituents of the nutrient medium used, and the addition of 0·2 per cent. glucose sometimes resulted in the growth of organisms less type-specific than cocci grown without glucose. In cultures grown at 22° C. both M and T antigens were invariably exposed. That the T antigen was present, though recessive, in type-specific 37° C. cultures was shown by their production of T antibodies when injected

into rabbits (*vide infra*). The type 24 strain 1364 lacked the T antigen but possessed the type-specific M substance; it existed only in the M or type-specific phase.

The strains representative of Type 4 so far examined appear to possess only the T antigen common to Types 4 and 24. In the Lancefield classification they would presumably be regarded as glossy variants of Type 24, although the strain Wheatley produced coherent colonies with a matt surface when grown on horse blood agar. This strain had been kept for some years in subculture and was not virulent for mice after 30 passages. It may not, therefore, be representative of all Group A Type 4 streptococci, some of which may later be shown to possess a Type 4 specific antigen.

Lancefield (1928) has described the M substance as being readily digested by trypsin. This was confirmed here by the action of trypsin on G25 and 1364 in the type-specific or M phase. The T antigen differs from the M substance in being resistant to trypsin but inactivated by heating to 100° C.

THE RELATIONSHIP BETWEEN TYPE 14 AND PROVISIONAL TYPE R491.

In investigating this relationship the strains Lowe (Type 14) and R491, both originally isolated and classified by Griffiths, were used in the preliminary experiments later confirmed with other strains belonging to these two types. Antisera were prepared, and agglutination, precipitation and absorption tests were carried out in the manner already described. The results of these tests are set out in Table III.

TABLE III.—*Reactions of Lowe (Type 14) and R491 (Provisional Type) with Absorbed Sera Showing Titre of Residual Agglutinins.*

	Serum.					
		Lowe (Type 14) absorbed with Type 3 non-specific and—				R491 (provisional type) absorbed with Type 3 non-specific.
*Agglutinating suspensions and "M" extracts.	Lowe absorbed with Type 3 non-specific.	Lowe (37° C.).	Lowe (22° C.).	R491 (22° C.).	Lowe "M" extract.†	
Lowe (37° C.)	30	—	—	30	—	—
,, (22° C.)	1920	960	—	120	960	+
,, (37° C.) trypsinized	—	—	—	—	—	—
,, (22° C.) ,,	1920	960	—	—	960	+
,, (37° C.) boiled	60	—	—	30	—	—
,, (22° C.) ,,	30	—	—	30	—	—
,, "M" extract	+	—	—	+	—	—
R491 (37° C.)	+ or —	+ or —	—	—	+ or —	+ or —
,, (22° C.)	960	960	—	—	960	+
,, (37° C.) trypsinized	+ or —	+ or —	—	—	+ or —	+ or —
,, (22° C.) ,,	++	++	—	—	++	++
,, (37° C.) boiled	—	—	—	—	—	—
,, (22° C.) ,,	—	—	—	—	—	—
,, "M" extract	—	—	—	—	—	—

The figures shown in the serum columns represent the reciprocals of the titres to which the suspensions were agglutinated on the slide.

* Agglutinating suspensions were grown in digest broth with 0·2 per cent. of added glucose. They were incubated at 37° C. or 22° C., and after growth some were digested with trypsin or heated to 100° C. All were then resuspended in normal horse serum (see text).

† The titration figures for residual agglutinin in Lowe serum absorbed with Lowe "M" extract were obtained after only one absorbing dose which removed M without affecting T antibodies. A second absorption of this sample of serum with "M" extract reduced the "T" antibody titre to 1/120.

It will be seen that between these two strains a relationship exists similar to that described between Types 4 and 24. Lowe (Type 14) appears to exist in at least two phases: One, type-specific, is found, although not invariably, in cultures grown at 37° C., which are agglutinated only by homologous sera; the other occurs in cultures grown at 22° C., which are always agglutinated by both homologous and R491 sera. The type-specific antigen is digested by trypsin to which the common antigen is resistant. The latter is inactivated by heating to 100° C., which is apparently without effect on the type-specific antigen. Type-specificity depends upon the presence of the M substance, while the common factor probably belongs to the group of T antigens.

A type-specific antigen has not yet been demonstrated in strain R491. Strains of this type might, therefore, be considered as glossy variants of Type 14, although, as in the comparable case of Wheatley (Type 4), the cultures were made from colonies considered to have "matt" characteristics on blood agar. Some suspensions made from R491 cultures grown at 37° C. were not agglutinated by sera of homologous or any other type. The reason for this is unknown. It is possible that such cultures were in a type-specific phase for which the corresponding antibodies were lacking in the homologous serum. Repeated attempts to prepare such a type-specific agglutinating serum have failed, and precipitation tests with M extracts prepared from R491 were invariably negative.

Difficulties Encountered with Type 14.

In this investigation difficulties arose which had not been encountered in the 4/24 experiments. Cultures of Lowe and other Type 14 strains, when grown in heart digest broth at 37° C., were granular. Cultures grown at 37° C. suitable for agglutination tests were obtainable only in serum broth or by resuspending in normal horse serum the deposit from a simple nutrient broth culture. Suspensions prepared by the latter method were used in these experiments. 22° C. cultures in all media yielded satisfactory suspensions, but for the sake of uniformity when 37° C. and 22° C. cultures were being compared both were suspended in normal serum.

Type specificity was not invariable in Lowe cultures grown at 37° C. In the comparable instance of G25 (Type 24) a reliable culture with constant type specificity at 37° C. was obtained by picking a large number of colonies into broth, and selecting a culture which remained type-specific after 15 to 20 subcultures. With Lowe this method failed to yield a culture which could be relied upon to produce type-specific suspensions, and it was usually necessary to put up several subcultures and select one.

Investigation of Type 14 and provisional Type R491 strains other than Lowe and R491.

Among the type strains left by Dr. Griffith there were three in addition to Lowe belonging to Type 14; these were Barker, Q7T and F69. They have been found to possess the properties described as characteristic of Lowe. Each possesses M and T antigens, of which the latter is shared with R491 and is exposed in cultures grown at 22° C., while the former is type-specific and dominant in 37° C. cultures, but appears together with the T antigen in 22° C. cultures. During the examination of more than 3000 strains submitted for routine typing during the last two years I have encountered only one which possessed both M and T antigens characteristic of Type 14. Streptococci with the Type 14 T antigen have been met with frequently, and these have been assigned for the time being to provisional Type R491.

Type 14/R491. Summary.

Type 14 is similar to Type 24 in possessing both M and T antigens. The M antigen is specific and may be dominant in cultures grown at 37° C., which are then agglutinated only by Type 14 antisera. The T factor is shared with provisional Type R491 and appears with the M antigen in cultures grown at 22° C., which are agglutinated by both Types 14 and R491 antisera.

Provisional Type R491 is similar to Type 4 in showing no evidence of a type-specific or M antigen. Cultures of this type grown at 37° C. were sometimes not agglutinated by absorbed sera of any type: the reason for this is unknown. 22° C. cultures are agglutinated by both homologous and Type 14 sera by virtue of the common T antigen.

These M and T antigens have similar properties with respect to heat and tryptic digestion as have those concerned in the 4/24 relationship.

The immunization of rabbits with heated and trypsin digested cultures.

Rabbits were inoculated with formolized cultures of strains belonging to Types 4, 24, 14 and R491. The cocci were grown in digest broth at 37° C. for 18 hours, after which they were centrifuged and resuspended in saline, giving a concentration of about 1000 million cocci per c.c. In some cases they were then heated to 100° C. for 15 minutes; in others they were digested with trypsin at 37° C. Before use formalin was added to all the vaccines to give a final concentration of 0·2 per cent.

Starting one week after a sensitizing dose of 1 c.c. of culture, each rabbit received, weekly, 3 intravenous injections for 4 weeks. During the first week 0·5 c.c. of bacterial suspension was given at each inoculation, and thereafter the doses were increased each week by 0·5 c.c. of suspension. The rabbits were bled each week and the sera absorbed with a non-specific Type 3 suspension. The absorbed samples were titrated against suspensions of Types 4 and 24 or 14 and R491. The final titres are shown in Tables IV*a* and IV*b*.

It will be seen that rabbits inoculated with cocci of Type 24 (rabbits 64 and 68) or Type 14 (rabbits 77 and 83) in the type-specific phase (M antigen dominant, T antigen recessive) produced both M and T antibodies. Strain 1364 (Type 24) was type-specific and produced only M antibodies in rabbit 91, but it will be recalled that this streptococcus showed no evidence of possessing a T antigen and remained in the M phase under all conditions of cultivation. Boiled type-specific cultures of Type 24 (rabbits 67, 93, 107, 108 and 110) and Type 14 (rabbits 78, 105 and 106) produced M but no T antibodies. Trypsin-digested type-specific cultures of Type 24 (rabbit 63) and Type 14 (rabbits 76 and 97) produced T but no M antibodies.

Results with the non-type-specific cultures Wheatley and 1337 (Type 4) and R491 and 1358 (Type R491) were less clear cut. Neither agglutination nor precipitation tests had given evidence of a type-specific antigen in these cultures, which were therefore thought to possess only the T antigen shared with Type 24 in the case of Wheatley and 1337 and with Type 14 in the case of R491 and 1358. Untreated or trypsin-digested vaccines produced T antibodies to a high titre in rabbits 65 (Type 4), 80 and 81 (Type R491), but rabbit 82 (Type R491) failed to respond. Boiled vaccines, no longer agglutinated by T antisera of corresponding type, produced neither M nor T agglutinins in rabbits 103 and 104 (Type R491), and T antibodies to low titre in rabbits 73 and 90 (Type 4) and 79 (Type R491).

Rabbit 78 (Type 14), which was originally inoculated with boiled vaccine and produced a type-specific serum, was given a second course of injections commencing three months after the bleeding shown in Table IV*b*. Serum taken after this second course was found to be no longer type-specific, but agglutinated R491 to a titre of 1/160.

Type 1.

Among the type cultures left by Dr. Griffith there were two belonging to Type 1: SF130/2, against which a "typing" serum had been prepared, and Smithers, a

TABLE IV*a*.—(*Types 4 and 24.*) *Comparison of Agglutinating Sera Produced by Rabbits Immunized with Untreated, Trypsin-digested, or Boiled Vaccines. Final Titres of Sera after Absorption with Type 3 (Non-specific).*

Rabbit number.	Formolized vaccine.	Final titre of sera against agglutinating suspension of— Wheatley (37° C.) (Type 4).	G25 (37° C.) (Type 24).
64	G25 (37° C.) (Type 24 specific)	640	80
68	,, (,,) (,, ,,)	320	40
63	,, (,,) trypsinized	320	—
67	,, (,,) boiled	—	40
107	,, (,,) ,,	—	240
108	,, (,,) ,,	—	120
110	,, (,,) ,,	—	240
91	1364 (37° C.) (Type 24 specific)	—	80
92	,, (,,) trypsinized	—	—
93	,, (,,) boiled	—	80
65	Wheatley (37° C.) (Type 4)	5120	—
73	,, (,,) boiled	20	—
90	1337 (37° C.) (Type 4) boiled	320	—

TABLE IV*b*.—(*Type 14 and Provisional Type R491*). *Comparison of Agglutinating Sera Produced by Rabbits Immunized with Untreated, Trypsin-digested, or Boiled Vaccines. Final Titres of Sera after Absorption with Type 3 (Non-specific).*

Rabbit number.	Formolized vaccine.	Final titre of sera against agglutinating suspensions of— Lowe (37°C.). Type 14.	Lowe (22°C.).	R491 (22°C.).
77	Lowe (37° C.) (Type 14 specific)	30	1920	960
76	,, (,,) trypsinized	..	+	+
97	,, (,,) ,,	—	480	480
78	,, (,,) boiled	40	—	—
106	,, (,,) ,,	60	—	—
83	Q7T (37° C.) (Type 14 specific)	20	160	80
105	,, (,,) boiled	480	—	—
104	R491 (37° C.) ,,	—	—	—
82	,, (,,)	—	—	—
81	1358 (37° C.) (Type R491)	—	480	960
103	,, (,,) (,,) boiled	—	—	—
79	,, (,,) (,,) ,,	—	60	120
80	,, (,,) (,,) trypsinized	—	480	1920

The figures shown in the serum columns represent the reciprocal of the titre to which the suspensions were agglutinated on the slide.

non-haemolytic streptococcus belonging to Group A and classified by Griffith as Type 1. The reactions of these two strains with the SF130/2 serum are shown in Table V.

Interpreting these reactions in the light of the experience gained with Types 24 and 14, it appears that in its present state SF130/2 has a dominant M antigen in cultures grown either at 37° C. or 22° C. This antigen is heat-resistant but digested by trypsin. It can be extracted from the bacteria in the usual manner, and these extracts are precipitated specifically by SF130/2 antiserum. There appears to be no T antigen in this culture of SF130/2. The strain Smithers possesses both M and T antigens

TABLE V.—*Agglutination Reactions of Type 1 Suspensions with SF130/2 (Type 1) Serum.*

Agglutinating suspension.	Serum. SF130/2 absorbed with Type 3 non-specific.	SF130/2 absorbed with Type 3 (non-specific) and— SF130/2 (37° C.) (Type 1).	Smithers (37° C.) (Type 1).	Smithers (22° C.) (Type 1).
SF130/2 (37° C.)	60	—	—	—
,, (,,) trypsinized	—	—	—	—
,, (,,) boiled	120	—	—	—
,, (22° C.)	120	—	—	—
,, (,,) trypsinized	—	—	—	—
,, (,,) boiled	60	—	—	—
Smithers (37° C.)	1920	480	—	—
,, (,,) trypsinized	480	480	—	—
,, (,,) boiled	120	—	—	—
,, (22° C.)	960	960	—	—
,, (,,) trypsinized	480	480	—	—
,, (,,) boiled	—	—	—	—

The figures shown in the serum columns represent the reciprocals of the titres to which the suspensions were agglutinated on the slide.

specific to Type 1. The T antigen is dominant in cultures grown at 22° C., while both M and T antigens contribute to the agglutination of cultures grown at 37° C.

Absorption of SF130/2 serum with Smithers grown either at 22° C. or 37° C. removed both M and T agglutinins. This absorption of M agglutinins by a suspension in which the corresponding antigen was recessive is not in agreement with the results of similar experiments with Types 24 and 14.

The presence of T agglutinins in the SF130/2 serum and the failure to demonstrate the corresponding antigen in cultures of this organism may be explained by assuming that SF130/2, isolated by Griffith some years ago when the antiserum was prepared, has since changed, losing the T antigen but retaining the M substance. Attempts to immunize rabbits with the organism in its present state have resulted in the production of M antibodies only. Both heated and unheated vaccines were effective; trypsin digested vaccines produced only non-specific antibodies.

DISCUSSION.

The results reported here confirm the work of Lancefield (1940*a*) in showing that more than one antigen-antibody system may operate in the agglutination of certain Group A streptococci by homologous antiserum from which general non-specific antibodies have been removed by absorption. For the sake of uniformity Lancefield's designation of these antigens as M and T substances has been conformed to here.

Lancefield (1928) has described the extraction of the M substance from streptococci in the " matt " phase, and she has found that this antigen is readily digested by trypsin. In the types studied here the T antigen has been distinguished by its resistance to tryptic digestion and its destruction by a temperature of 100° C. to which the M substance is resistant. The general non-specific Group A antigens are resistant both to 100° C. and to overnight tryptic digestion. The nature of the dominant type antigen or antigens in these cultures has thus been readily determined.

In identifying these antigens by slide agglutination tests it appears that *in these types* the M antigen is most readily distinguished in cultures grown at 37° C. and the T antigen by growth at 22° C. Organisms possessing both antigens may, therefore,

be subject to a form of diphasic variation in which either the M or T antigen may be dominant, depending on the conditions of cultivation. Strains belonging to Types 14 and 24 have been found in the M phase when grown at 37° C., while both M and T antigens appeared in cultures grown at 22° C. The strain Smithers (Type 1) was in the T phase when grown at 22° C., and exhibited both M and T antigens when grown at 37° C. In diphasic strains, when one antigen is dominant the other is recessive, and rabbits immunized with such cultures produce antibodies corresponding to both antigens. Some more recent observations suggest that a similar phenomenon occurs with some diphasic strains belonging to Type 5. It is of interest to recall that antigenic variation determined to some extent by the temperature of incubation has been noted in the genus *Pasteurella* by Schutze (1932).

Monophasic variants of these diphasic types have been encountered. The variant in which the M antigen is absent has been described by Lancefield as occurring in the glossy variants of matt strains (1940*a*). The other form in which the T substance is lacking has been met with in this investigation. Strain 1364 (Type 24), when freshly isolated, gave no evidence of possessing a T antigen either in agglutination tests or rabbit immunization experiments. The same might be said of SF130/2, Griffith's Type 1 strain, although the organism has apparently lost its T antigen during subculture over a number of years.

In addition to this varied antigenic constitution in strains of a given type, there is some evidence that the association between individual M and T antigens is by no means constant. Lancefield (1941*b*) has described a strain C203 in which the M and T antigens of Type 3 are associated with the T substance of Type 1. In the work described here the T antigen of Type 24 has been found in Type 4 cocci, while that of Type 14 is shared with provisional Type R491. The slide agglutination technique was used by Griffith for identifying the *dominant* type antigen in the streptococcal cultures examined by him (Griffith, 1935). It now appears that for the precise identification of any strain a full analysis of its M and T components is necessary if errors in classification are to be avoided.

It would be misleading to create the impression that the full identity of the type antigens is always to be readily determined by the examination of boiled or trypsin digested suspensions in the slide agglutination test. It has been a matter of good fortune that the strains described here have yielded clear-cut results by this technique, but it is not to be expected that all types will be as readily identified. Some of the difficulties have already been mentioned. Others exist and may be briefly summarized.

Strains are frequently encountered which fail to give usable suspensions, especially when grown at 37° C. Methods designed to improve these suspensions may modify the naturally occurring dominant antigen. This applies particularly to tryptic digestion and variations in the temperature of incubation during growth. After boiling or digesting to destroy the T or M antigens, some suspensions, previously good, become either granular or sensitive to normal saline or serum. Other strains are encountered which for some reason yield inagglutinable suspensions. This may sometimes be due to their being in either a " mucoid " or a non-specific phase ; non-specific variants of Types 3, 13 and 19 have been described by Griffith (1935). The difficulty of maintaining the type specificity of some diphasic strains in subculture has already been mentioned. In addition to the temperature of incubation, the constituents of the nutrient media and especially the presence of glucose appears to affect the dominant antigens. It is of some interest that Griffith deprecated the use of glucose broth because of its tendency to induce " groupiness " of the agglutinating suspension. By " groupiness " he usually meant the susceptibility to non-specific agglutinins normally removed from the " typing " sera by absorption with hetero-

logous strains. It now appears likely that part, at any rate, of this " groupiness " was due to the unmasking of T antigens in the agglutinating suspensions.

Difficulties may also be encountered in the production of " typing " sera having the full complement of M and T antibodies representative of the type antigens. Some of the Griffith sera have been found deficient in this respect, and their use might well lead to errors by identifying part only of the full antigenic make-up of the strains under examination.

The Griffith series of type strains were, in a sense, obtained by a process of selection. A full knowledge of their antigenic constitution is the framework about which a knowledge of the taxonomy of *Streptococcus pyogenes* may be built. It is possible, however, that the corresponding M and T antigens are distributed in a more or less random fashion amongst the streptococci of this species, giving rise to intermediate types possessing the antigens of more than one of the classical type strains. The Lancefield strain C203 is an example of this, and it is common knowledge among those working on this problem that strains giving fresh conbinations of agglutination reactions with the Griffith type sera are constantly being encountered. The epidemiological relationship of these intermediate types to the classical types where both are isolated during the course of the same outbreak is not yet understood, but it is of obvious importance.

It follows from all this that for the serological identification of *Streptococcus pyogenes* a full knowledge of the " typing " sera and a comprehensive experience of the vagaries of streptococcal agglutinating suspensions are essential. In inexperienced hands the slide agglutination technique used in the identification of these organisms may lead to most misleading results.

SUMMARY.

1. Group A streptococci belonging to Types 24, 14 and 1 have been subjected to crude antigenic analysis. In addition to general non-specific antigens they possess both the M and T antigens described by Lancefield.

2. The T antigens of Types 24 and 14 are shared with Types 4 and provisional Type R491 respectively.

3. Strains possessing both M and T antigens may be subject to a form of diphasic variation, either antigen being dominant when the other is recessive. At other times both antigens appear together. In the types studied here the M phase appeared to be favoured by growth at 37° C.; T antigens were unmasked by growth at 22° C. and sometimes by incorporating in the broth 0·2 per cent. of glucose.

4. The M substance has been shown by Lancefield to be resistant to heat (100° C.) and to be digested by trypsin. The T antigens appear to be resistant to tryptic digestion but inactivated by exposure to 100° C.

5. The use of boiled or digested vaccines is described in the production of antisera containing either M or T antibodies respectively.

6. Antigenic variants of the types studied here are described and the difficulties encountered in their identification discussed.

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